THE INFLUENCE OF DIET ON TRANSPLANTED AND SPONTANEOUS MOUSE TUMORS.*

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PLATE 21.

That cancer progresses relatively slowly in emaciated, old people has long been known; but the influence on the disease of general nutritive conditions as thus indicated has only lately attracted wide attention, following the observation that some strains of transplantable tumors grow badly, or not at all, in emaciated hosts. Moreschi[1] was the first to study the phenomenon systematically. He found that grafts of mouse sarcoma grew less frequently and more slowly in animals losing weight on a low diet. But, though he thus demonstrated that malnutrition of the host affects adversely bits of strange tissue as yet unvascularized and unattached, he did not take up the influence of the factor on large growths, on recurrences, and on the development of metastases, or, in other words, on neoplastic conditions such as require palliative treatment in human beings. Experiments in this direction which I began[2] shortly after Moreschi's paper was published demonstrated the fact that a tumor which does badly when transplanted to hosts previously underfed may be quite uninfluenced by dieting begun after the growth is vascularized and has started to develop. Nodules of the Flexner-Jobling rat carcinoma, a few millimeters in diameter, grew with equal rapidity in hosts emaciating on a restricted ration and in controls gaining weight on the same sort of food (text-figures 1 and 2). The Jensen rat sarcoma, on the contrary, even after it had reached a large size, was retarded in its development by under-

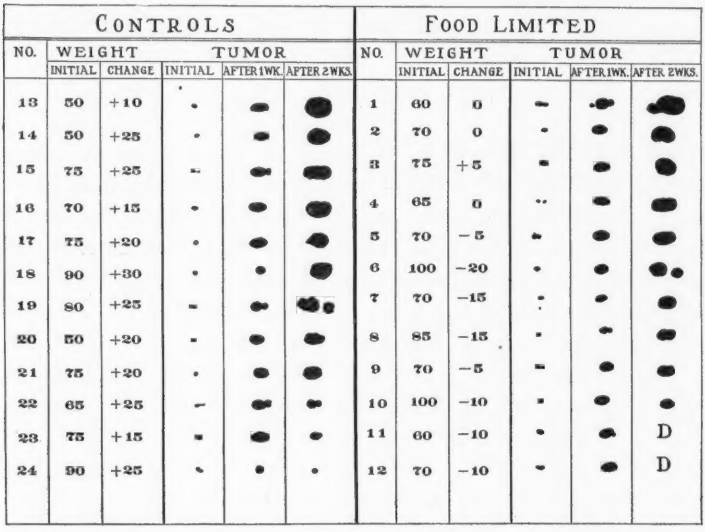
* Received for publication, August 1, 1914.

[1] Moreschi, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, ii, 651.

[2] Rous, P., *Proc. Soc. Exper. Biol. and Med.*, 1910–11, viii, 128.

feeding the host. But the forces which determine the course of this tumor must be delicately balanced, for it often retrogresses without evident cause in whole series of healthy rats.

Not only have general nutritive conditions an influence on some transplanted tumors, but the character of the host's food may be important. Van Alstyne and Beebe[3] have shown that tumor grafts



TEXT-FIG. 1. The Flexner-Jobling adenocarcinoma, once it has begun to develop, grows with equal rapidity in rats gaining weight on a mixed diet and those underfed on the same food.

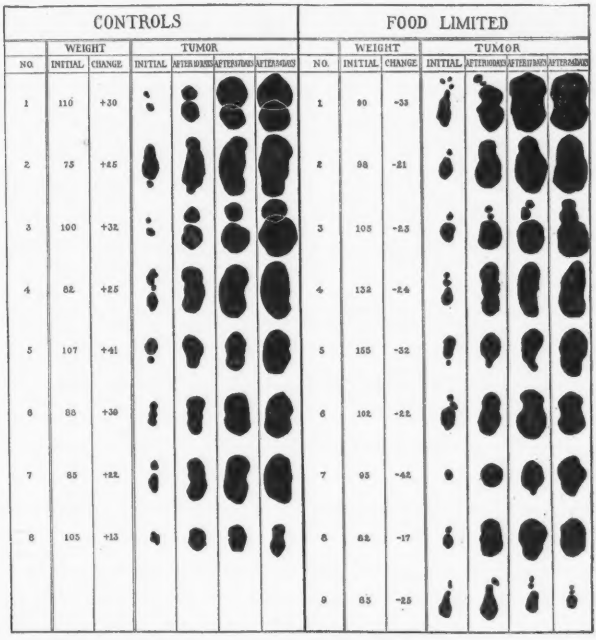
give rise relatively seldom to growths in animals fed for some time beforehand on a non-carbohydrate diet. Sweet, Corson-White, and Saxon[4] kept mice on a modification of a food devised by Mendel and Osborne[5] that prevents the growth of rats because of the lack of certain constituents; and they found that a mouse car-

[3] Van Alstyne, E., and Beebe, S. P., *Jour. Med. Research*, 1913–14, xxix, 217.

[4] Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *Jour. Biol. Chem.*, 1913, xv, 181.

[5] Osborne, T. B., Mendel, L. B., and Ferry, E. L., *Ztschr. f. physiol. Chem.*, 1912, lxxx, 307.

cinoma had poor success in such hosts. Unfortunately it is not certain whether the results of these investigations are to be attributed to a specific lack in the foods employed or to the circumstances that the diet of the specially fed hosts differed from that of the animal furnishing the tumor transplanted to them. That such a difference may adversely affect tumor grafts has repeatedly been shown[6]; and



TEXT-FIG. 2. This records findings similar to those of text-figure 1. But the dieting was begun when the tumors were larger and was continued for a longer time. The underfed animals lost greatly in weight.

it was with tumor grafts that the investigators mentioned obtained their positive results. Van Alstyne and Beebe found a non-carbohydrate diet ineffective when it was begun on the day that the tumor was implanted. Sweet and his collaborators made experiments only with tumor grafts.

[6] Haaland, M., *Berl. klin. Wchnschr.*, 1907, xliv, 713. Stahr, H., *Centralbl. f. allg. Path. u. path. Anat.*, 1909, xx, 628.

Altogether the work mentioned shows that the development of tumor grafts can in many cases be prevented or retarded by underfeeding the host or by putting it on a special diet. By the first method certain transplantable tumors can be checked or retarded in their course even after they have reached a large size. The method is ineffectual with established growths of one kind at least, the Flexner-Jobling rat carcinoma, despite the fact that grafts of this tumor, as yet unvascularized, are easily influenced. Whether recurrences and metastases of such a growth will behave like the parent tumor, or, like new made grafts, prove sensitive to influences exerted through the diet is not yet known; nor has the influence of special diets on large tumors been adequately looked into. Apart from certain suggestive clinical instances nothing is known as to the influence on spontaneous growths of alterations in the food of the host. The present study is concerned with some of these points.

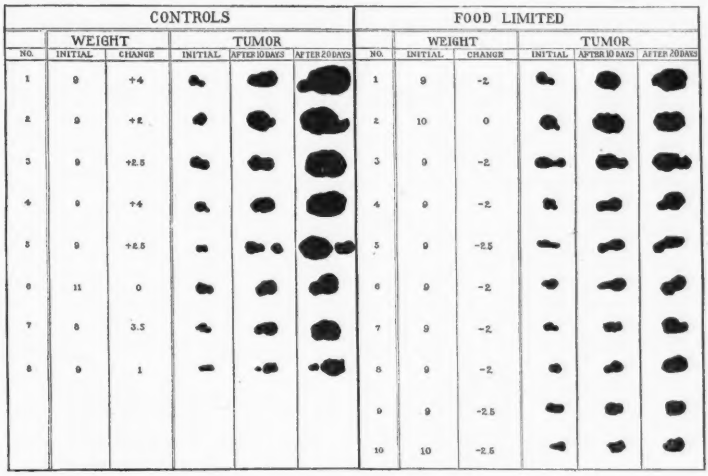
THE INFLUENCE ON TRANSPLANTED TUMORS.

Several mouse tumors and the Flexner-Jobling adenocarcinoma of the rat were used. The mice which served as hosts were not merely underfed so that they lost weight but were given as their sole diet Sweet's modification of Mendel's food.[7] It was thought that in this way more outspoken results might be obtained. Rats, as in previous experiments with the Flexner-Jobling growth, were underfed on a standard bread compounded of oatmeal, corn-meal, rye flour, milk, and sugar with a measured quantity of fresh milk to moisten it. Experiments later to be described show that Sweet's food has no special influence on this tumor.

In the attempt to learn whether the development of metastases can be influenced by diet, experiments were carried out with two metastasizing mouse carcinomata, kindly furnished by Dr. Tyzzer and by Dr. Bashford, respectively. One was a growth of the Japanese waltzing mouse, the other strain 63 from the laboratories of the Imperial Cancer Research Fund. But as both these tumors, even after they had reached a considerable size, were markedly checked in their growth by underfeeding the host on Sweet's

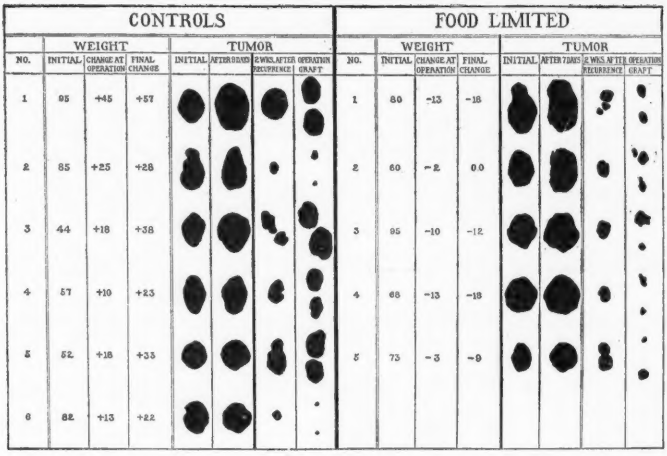
[7] A food composed of starch, lard, lactose, agar, a salt mixture, and gluten obtained by washing wheat flour.

food (text-figure 3), it seemed useless to observe the effect of the treatment on secondaries, since the number of these secondaries is of course directly dependent on the activity of the primary neoplasm. The Flexner-Jobling tumor could not be utilized because it had ceased to metastasize with sufficient regularity. A strain of mouse carcinoma (native strain No. 33) which never gives secondaries, was tested and found to be markedly held back by the dieting.

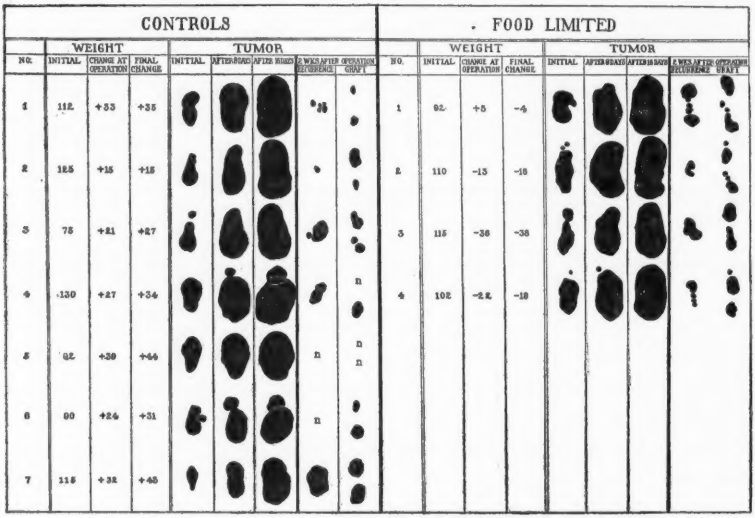


TEXT-FIG. 3. A transplantable adenocarcinoma of the Japanese waltzing mouse was markedly retarded in its growth by underfeeding the host on Sweet's food. The diet was begun after the tumors had reached a considerable size. The controls were full fed on a mixed ration.

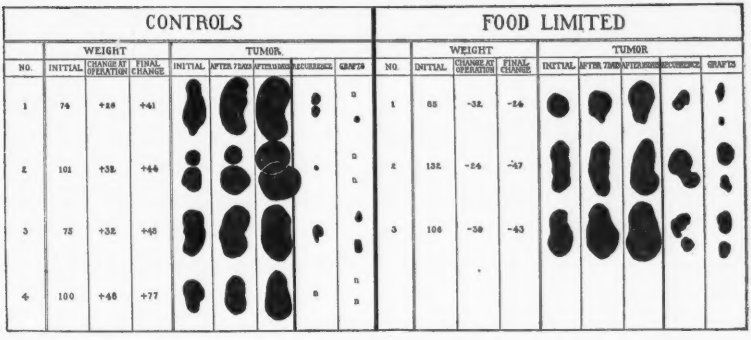
The effect of underfeeding upon recurrences was studied with the Flexner-Jobling adenocarcinoma. Just as three years previously, this growth was uninfluenced by changes in the nutritive condition of the host instituted after it had reached considerable size (text-figures 4, 5, and 6). Rats about three fourths grown with subcutaneous tumors resulting from inoculation of the same material were paired against one another according to body-weight and size of the tumors, and one of each pair was underfed so that it grad-



TEXT-FIG. 4.



TEXT-FIG. 5.



TEXT-FIG. 6.

TEXT-FIGS. 4, 5, AND 6. These give the results of three experiments with the Flexner-Jobling adenocarcinoma, designed to test the effect of underfeeding on recurrences and operative disseminations (grafts) of this growth. Half of the animals were underfed on a mixed diet, half full fed; and after several weeks all were operated upon. The tumor was removed save for a small fragment, and two auto-implantations were made in the subcutaneous tissue.

In one series of underfed animals (text-figure 4) the recurrences and grafts grew less well than in the controls; in a second series (text-figure 5) no notable differences were observed; while in the third growth was far better in the underfed animals.

ually lost in weight. The controls were full fed on the same diet and grew rapidly. After several weeks of dieting, when the experimental animals had become thin, all were operated upon by a uniform procedure. The tumor was removed save for a fragment about one and one half millimeters in diameter, which was left with its vascular connections intact; and two autotransplantations were made of small bits to the subcutaneous tissue of the flanks. The feeding was not changed. The results varied strikingly from experiment to experiment. In one series of thin animals the development of recurrences and grafts was much delayed, in another it occurred with the same rapidity as in the controls, and in a third it was much more rapid (text-figures 4, 5, and 6).

Evidently in these experiments with the Flexner-Jobling tumor factors sufficient to cause a complete reversal in the findings were uncontrolled. Without doubt one such factor is the resistance elicited in the host by the implanted alien tissue. This resistance

has been often studied. That it may lead to the retrogression of transplanted tumors, even when large, or prevent recurrences of them is well known. Through the instability that it introduces it may have been responsible for the sensitiveness to alterations in the host's diet shown by the transplanted mouse tumors used in the present work. Against spontaneous tumors such a resistance has not been demonstrated despite repeated attempts. So the findings with transplantable tumors can hardly be taken as a basis for generalizations regarding the effect of diet on spontaneous growths.

THE INFLUENCE ON SPONTANEOUS TUMORS.

Some hundred and fifty spontaneous mouse tumors were obtained for the work. All had arisen as lumps in the mammary glands of old females, and all except three were carcinomata, these three being examples of carcinoma sarcomatodes. The histology of the growths was varied.

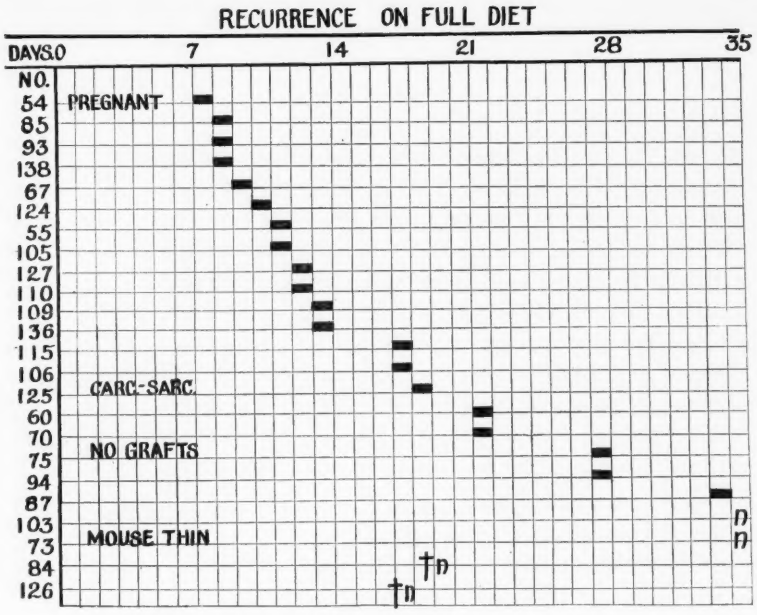
The tumor mice were divided into three groups of the same general character. One group which served as control was full fed on ordinary diet (bread and milk, grain); a second was underfed on Sweet's modification of Mendel's food for some days prior to operation and thereafter throughout the term of observation; and the third was given ordinary diet before operation, and an abundance of Sweet's food afterwards. Each animal had a jar to itself, provided with a water bottle. As in the experiments with the Flexner-Jobling carcinoma the tumors were removed save for a fragment about one and one half millimeters in diameter which was left with its vascular connections intact; and two autotransplantations of similar bits were made into the subcutaneous tissue of the animals' flanks. These latter bits may be taken to represent in some sort disseminations at the time of operation. The wounds were closed with very fine silk and usually healed by first intention. When there were multiple tumors, as frequently happened, all save one were taken out *in toto,* and a fragment of this one was left and implantations made as usual. The mice were kept for thirty-five days after operation and weighed and examined frequently. Haaland[8] has found

[8] Haaland, M., *Fourth Scientific Report of the Imperial Cancer Research Fund,* 1911, 1.

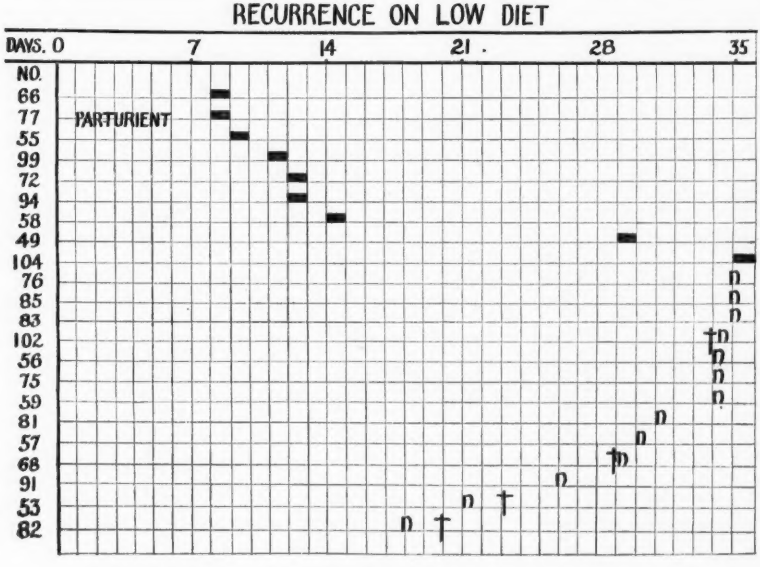
that grafts of spontaneous mouse tumor implanted in the animal furnishing the growth usually began to develop within thirty-five days. Text-figure 9 confirms his findings.

Many complications and deaths occurred among the operated mice, diminishing the number that could be used for a comparison. Some of the animals had very large tumors and were already emaciated. These were ruled out. In the charts are figured the results in mice surviving two weeks or longer without wound infection, or severe illness, or gaping of the wound that would affect the recurrence. As was to have been expected, much the largest proportion of deaths from intercurrent causes was in the underfed mice. Constant vigilance was needed to ensure that the food they received was sufficient to support life, and yet so little that they lost in weight. Minor ills such as otitis, scabby skin, and conjunctivitis were about equally frequent in the three series. Recurrences and growth of the grafts were first recorded as such when growing nodules larger than the original tumor fragments had made their appearance. The recurrences usually developed from the tumor bit left in the wound; but often, especially in the control group, they were multiple, or diffuse in the scar. Owing to the way in which the operation was carried out tumor cells must have been scattered throughout the wound in practically every case.

A comparison of text-figures 7 and 9 with text-figures 8 and 10 shows that in the mice losing weight, as a result of underfeeding with Sweet's modification of Mendel's food, recurrences and the growth of tumor grafts were delayed in most cases and prevented in many throughout the term of observation. In 83 per cent. of the twenty-four mice on ordinary diet a recurrence was noted, and in only 41 per cent. of the twenty-two underfed mice. The effect on growth of the grafts was somewhat less marked. They grew in 68 per cent. of the controls and 41 per cent. of the underfed animals, appearing on the average fifteen days after operation in the former and twenty-five days after it in the latter. In the controls the new tumors enlarged much more rapidly, and coalescing recurrences throughout the healed wound were relatively frequent. A few of the tumors in the underfed animals,—and these the most malignant histologically,—were little if at all affected by the dieting,

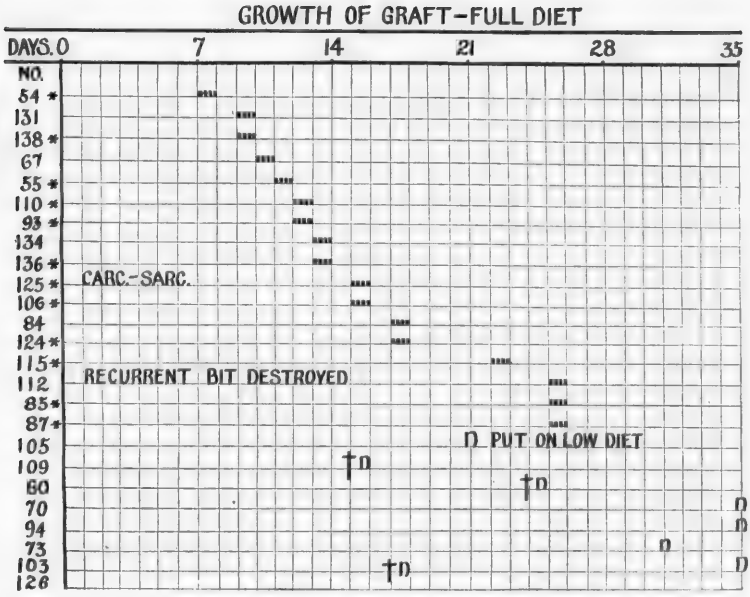


TEXT-FIG. 7.

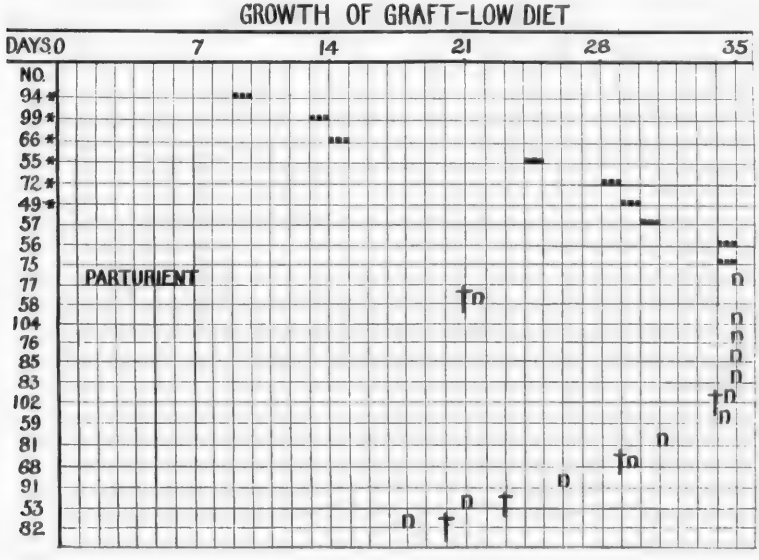


TEXT-FIG. 8.

TEXT-FIGS. 7 AND 8. These give the time of appearance of recurrences of the spontaneous tumors of a series of mice fed abundantly on a mixed diet, and of another series underfed before and after operation on Sweet's food. In the first column are the numbers designating the mice. The distance of the black dashes from the ordinate indicates how soon after operation the recurrences appeared. N = no recurrence.



TEXT-FIG. 9.



TEXT-FIG. 10.

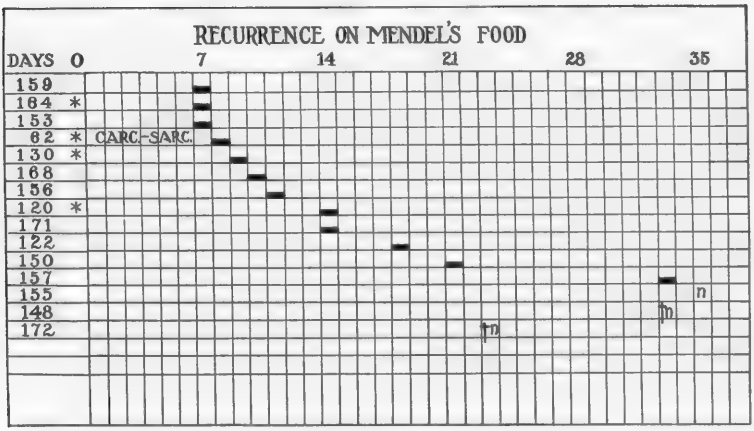
TEXT-FIGS. 9 AND 10. The time of appearance of growing grafts in the mice of text-figures 7 and 8. A star after the number of a mouse indicates that in this animal there was a recurrence as well.

as far as could be observed. Recurrences of these growths took place and grafts of them enlarged with practically the same rapidity as in the most malignant instances among the controls.

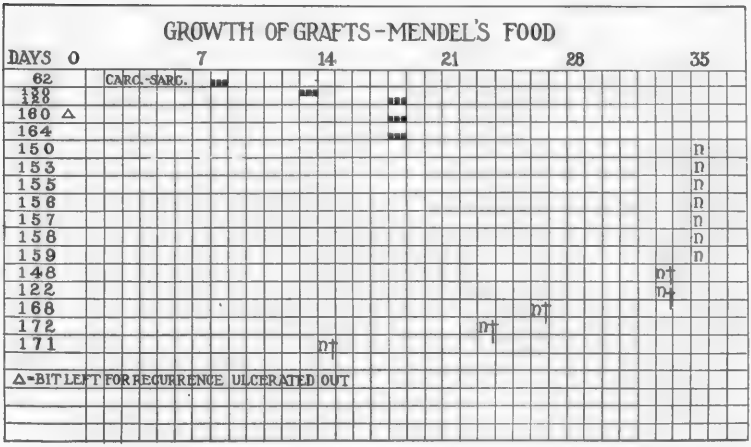
The treatment that secured these results in the dieted mice was drastic. By the time of operation the animals had lost, on the average, 12 per cent. of their weight and at the end of the thirty-five days 24 per cent. But as most were fat when obtained, the actual emaciation was less than is suggested by the figures. Toward the end of the fourth week the mice showed almost regularly an aversion for the special food, and it became necessary to supplement this with a little bread and milk if they were to remain alive.

The results were not permanent in that cures were effected. Some of the mice which showed no sign of tumor during the period of underfeeding were put on ordinary diet at its completion, and practically without exception they developed recurrences or growing grafts, or both, soon after regaining their weight. Some of the control animals in which the tumor had reappeared were transferred to the underfed group, and when they had begun to lose weight were operated upon a second time according to the usual procedure. Occasionally the results were striking. A carcinoma solidum which recurred on the control diet eight days after operation failed to reappear in the thirty-six days in which the host was underfed following a second operation, only to recur again, and within four days, when the control diet was resumed. In this animal the grafts did not grow.

It has been of special interest to determine whether results similar to those in the underfed group could be obtained in mice kept in nitrogenous equilibrium on the special diet. But in the work to this end an unexpected obstacle was met with. Rats stay in good health and retain their weight for long periods when fed on Mendel and Osborne's food; but on Sweet's modification of this as prepared in our laboratory mice were found to do badly. For example, nine healthy, adult mice weighing an average of twenty grams lost an average of 4.4 grams during three weeks in which they were given the food in abundance. Young animals fare even worse. Some old females maintain equilibrium for ten days or two weeks, but the majority begin to lose weight within a few days and by the



TEXT-FIG. 11.



TEXT-FIG. 12.

TEXT-FIGS. 11 AND 12. The time of appearance of recurrences and grafts, respectively, of the spontaneous tumors of mice fed abundantly on Sweet's food from the time of operation. In text-figure 11 a star after the number of a mouse indicates that the grafts also grew.

end of the fourth week are thin, cold, and weak, refuse the food and soon die unless supplied with another ration. All of the mice with spontaneous tumors, full fed on Sweet's food after operation, emaciated rapidly toward the end of the period of observation, their average loss of weight after thirty-five days being 30 per cent. or slightly more than that of mice underfed on the same diet.

Although the end results as regards loss of weight were nearly the same in the mice underfed and the mice full fed on Sweet's food, there were essential differences in the conditions imposed on the two groups of animals. The group which received an insufficient ration of the diet for some days prior to operation was losing weight when it was performed, whereas the mice of the other group were operated upon when still well nourished on an ordinary diet, and, being given thereafter an abundance of the special food, they lost little weight for some days. The effect of these differences is plainly to be seen in text-figures 11 and 12 which have to do with the results in the animals of the latter group. Recurrences in them (text-figure 11) took place as frequently and as soon as in the control animals (text-figure 7). Growth of the grafts (text-figure 12) on the other hand was delayed as greatly as in the underfed mice (text-figure 10).

There is a simple explanation for these facts. Under ordinary conditions (text-figures 7 and 9) the appearance of a recurrence precedes by a considerable period the development of grafts,—as would naturally follow from the circumstance that the bit left for a recurrence possesses a vascularization and other connection with the tissues, whereas the grafts must acquire this, a proceeding which takes some days. During these days the mice fed on Sweet's food had begun to lose weight, and the grafts on starting to grow had the factor of general malnutrition against them. The bits left for recurrences, on the other hand, were able to begin growing almost at once, at a time when the dieting had just been started, and the animals were still well nourished.

All in all the findings show that it is possible in many cases to hinder the development of bits of spontaneous mouse tumor disseminated at operation, by feeding the animal after operation on a special diet that entails a gradual loss of weight. In order to delay

the development of recurrences from tumor bits left *in situ* a more drastic treatment is required, namely, dieting that entails loss of weight previous to the operation.

THE NATURE OF THE INFLUENCE.

In what way are these results brought about? Has Sweet's modification of Mendel's food an influence to check the growth of tumors other than that exerted through the loss of weight that it entails? There is some reason to doubt this. Several experiments made with rats carrying the Flexner-Jobling tumor show that this growth is as unaffected by a diet of Sweet's food as by underfeeding on a mixed diet (text-figure 13). But to settle the point definitely

LOW DIET					SPECIAL DIET				
	WEIGHT					WEIGHT			
NO.	INITIAL	CHANGE	INITIAL	AFTER 3 WKS.	NO.	INITIAL	CHANGE	INITIAL	AFTER 3 WKS.
1	95	−23			1	85	0		
2	65	−18			2	72	−10		
3	57	−15			3	52	−1		
4	70	−16			4	77	−12		
5	47	−7			5	42	−5		

TEXT-FIG. 13. This shows that a diet of Sweet's food had no more effect upon the development of the Flexner-Jobling tumor than had underfeeding on a mixed diet.

tumors more sensitive to alterations in the general nutrition must be used.

The effects of the dieting can scarcely be to stimulate immunity processes in the host. For, as has been pointed out, the tumor cells are merely held in check, remaining alive and capable of rapid growth when the host is returned to ordinary food and regains its weight. In many instances, doubtless, the cells suffer directly from the general starvation of the body. There is reason to suppose, though, that most of the effect on them is indirect, through a limitation in the host's ability to form a connective tissue scaffolding and vascularization for their support. Whether the absence of this "stroma

reaction" is the essential factor in resistance to tumors, as assumed by Russell,[9] need not be here discussed. It is sufficient to know that all tumors and grafts of tumors, except those developing wholly by the invasion and replacement of normal structures, need in the course of their growth a stroma furnished by the host. In mice losing weight the proliferative activity of the host's tissues,—on which the elaboration of a stroma must ultimately depend,—is much decreased. This is shown, first by the extreme slowness with which wounds heal in these animals, and second by the delayed organization about inert, foreign bodies. I have found that clean, well apposed wounds frequently stay unhealed for a week or ten days. The encapsulation of agar-agar injected beneath the skin is also very slow.

Experiment 1.—Sixteen rats about three fourths grown were paired against one another according to weight, and half were full fed, half underfed on the same diet of mixed food. When the underfed group had begun to lose weight all were injected subcutaneously in each flank with 0.08 c.c. of 1.5 per cent. agar in Ringer's solution. The agar was cooled in the syringe, and forced through a small needle, whereby it was broken into many small fragments. It was kept from extending back along the injection track by pressure with a rubber-covered clamp. Thus each rat received in its subcutaneous tissue two discs or buttons of fragmented agar. Seven days after the injection the animals were killed and sections made through the center of each agar disc.

The reaction to agar has been often studied and need not be described further than to say that, following endothelial migration, strands of connective tissue extend among the bits of jelly which are at length wholly encapsulated and, little by little, penetrated and absorbed. These processes were found to be far advanced in the control animals of the present experiment, at a time when in the dieted ones a capsule about the agar was almost lacking and organization was just beginning (plate 21, figures 1 and 2). The experiment has been several times repeated, using both rats and mice, with the same results.

If the loss of weight caused by underfeeding retards the growth of tumors, how does it happen that tumors progress so rapidly in emaciating human beings? Probably in some instances because in

[9] Russell, B. R. G., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 341.

man, as in mice, certain tumors are able, *ab initio,* to obtain their food with ease in a starving body. It seems likely that certain other tumors during their development gain in the ability to obtain food through the survival and proliferation of the cells most suited to cope with the increasingly difficult conditions.[10] According to this idea a tumor made up at first of a mixture of cells sensitive and insensitive to alterations in the general nutrition would, as the host emaciated, come to consist of elements little affected by the circumstance. Some experiments were performed to test the hypothesis. Transplanted tumors which had grown for some weeks in full fed hosts and hosts underfed to the point of emaciation were transferred to underfed individuals. According to the premises, the tumors from the underfed hosts should have given the better results. But no differences were noted.

SUMMARY.

Previous work has shown that the growth of grafts of transplantable tumors can be in many cases prevented or retarded by underfeeding the new host or by putting it on a special diet. The effect of such treatment on large tumors has been little studied; and the effect on metastases and recurrences has not been studied at all. Apart from certain clinical observations nothing is known as to the influence on spontaneous tumors of alterations in the diet.

Experiments with transplanted rat and mouse tumors along the lines thus suggested show that large growths of certain strains are checked in their development by underfeeding the host upon a special diet (Sweet's modification of one of Mendel and Osborne's foods) or in some cases by simple underfeeding. Two metastasizing mouse tumors are instances in point. They stopped growing or grew very slowly in hosts underfed on the special diet. The Flexner-Jobling rat carcinoma, on the other hand, was unaffected by the most rigorous underfeeding on a mixed diet when this was begun after the tumor had been growing for a short period. Experiments

[10] Loeb has shown that tumors in mice treated with colloidal copper undergo regressive changes at first, but that a strain of cells unaffected by the drug may survive, from which the tumor eventually proliferates (Loeb, L., personal communication).

to test the influence of underfeeding upon recurrences of this tumor gave results that varied from series to series of animals. The findings strongly indicate that generalizations from work with transplanted tumors as regards the effects of diet on spontaneous growths are unwarranted.

By underfeeding on Sweet's food mice with spontaneous tumors, beginning some days prior to operation, it has proved possible in most cases to delay for a relatively long period the development of recurrences and the growth of tumor bits (grafts) disseminated at the time of surgical interference. The treatment entailed great loss of weight. Tumor mice kept on ordinary diet previous to operation, but put thereafter on an abundant ration of Sweet's food, developed recurrences as early as the tumor mice on ordinary diet; whereas the growth of auto-implants was, relatively speaking, much delayed. These results seem attributable rather to a gradual malnutrition induced by the special food than to the circumstance that it lacked a growth principle. In none of the dieted mice was a definite cure obtained. Ordinarily a recurrence appeared and the grafts began to grow soon after the host, again on ordinary food, had regained weight.

A few spontaneous tumors seem absolutely unaffected by the most rigorous dieting.

Wounds heal with marked slowness in animals that have become thin as a result of dieting, and an inert foreign body (agar-agar) injected subcutaneously is very slowly encapsulated and organized. In these facts may be found a suggestion as to the method whereby dieting delays tumor growth. For it may well be that, with a lessened proliferative activity of the host tissue, the elaboration of a vascularizing and supporting stroma such as most tumors depend upon for their growth, at least indirectly, is much delayed.

The rapid growth of tumors in emaciating individuals is not incompatible with the present findings. Such growth may be consequent upon a selection in the host of those cells most fit to cope with the increasingly difficult nutritive conditions. But experiments designed to demonstrate this have been unsuccessful.

It is conceivable that recurrences of certain human tumors and

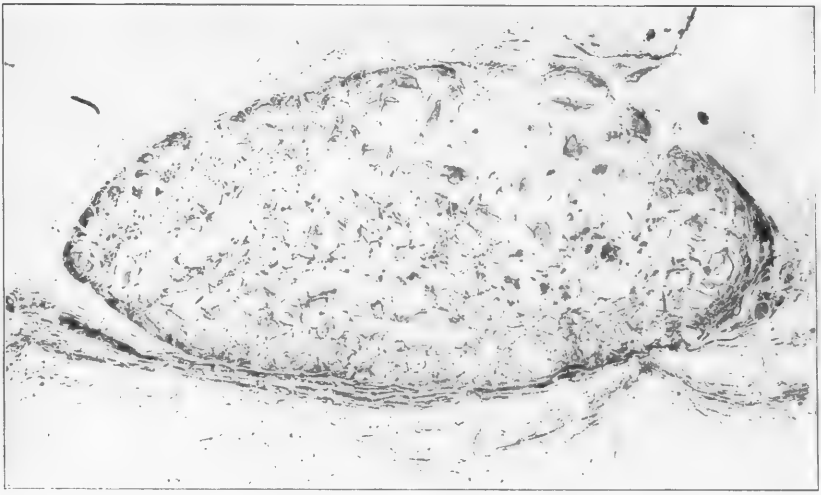


FIG. 1.

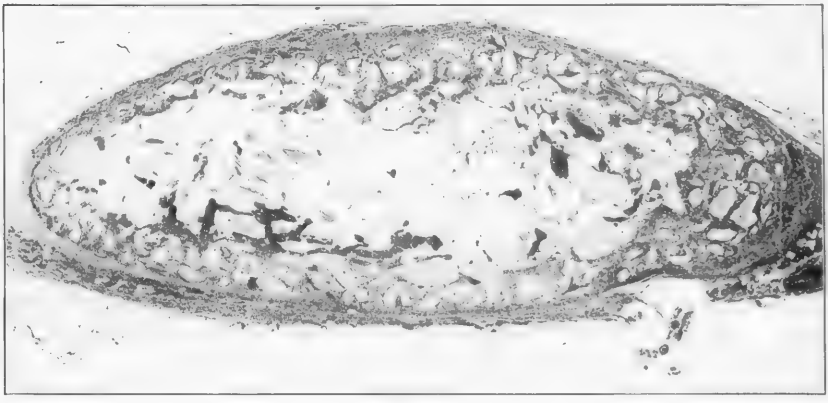


FIG. 2.

(Rous: Influence of Diet on Tumors.)

the development of metastases may be delayed or prevented for a period by methods somewhat similar to those employed against spontaneous mouse tumors in the present investigation. But generally speaking only the more malignant human tumors would require such palliative measures, and these are precisely the ones that would prove,—if experience with mice is an index,—least amenable to alterations in the nutrition of the host.

It is a pleasure to acknowledge the help of Dr. Linda Lange in this work.

EXPLANATION OF PLATE 21.

FIG. 1. Cross-section through an agar mass removed seven days after its injection into the subcutaneous tissue of a young rat which was losing weight as the result of underfeeding on a mixed diet. The encapsulation and organization are slight as compared with them in figure 2.

FIG. 2. Cross-section of an agar mass from a full fed control rat. Methylene blue and eosin.

BACTERIAL ANTIFERMENTS.

Studies on Ferment Action. XVII.*[1]

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The resistance of bacteria to enzyme action, because of its importance as a factor in the defense of the invading organism against the destructive agencies of the host, has interested several observers, among them Kruse (1), Fermi (2), Kantorowicz (3), and Weinkopff (4). Influenced possibly by the epochal advances in protein chemistry and by the fact that bacterial antigens are probably purely protein in character, workers in immunology have naturally investigated with great care the bacterial proteins, and the effect of proteolytic enzymes upon them.

The older conception that bacteria resisted digestion because of some vital property had to be discarded when it was found that organisms killed by chloroform, toluol, carbolic acid, etc., seemed to resist digestion as well as viable bacteria. It has so far been the implied idea that since bacteria contain protein, such protein should be an available substrate for enzyme action, as casein or edestin might be used; in other words, that the bacterial cell represented a naked and a freely exposed mass of protein. In dealing with an intact cell there are, however, certain factors which would indicate that this position is untenable.

Were we to assume that bacteria have a limiting membrane, such a membrane would, if an analogy to plant cells is permissible, contain no protein (Czapek (5)); if similar to animal cells they would probably be protected by a predominatingly lipoidal membrane (Meyer, Overton). There is, of course, no direct evidence, although with the exception of Hamm (6) there has been a uniformity in the reports of the absence of nitrogenous material even from the capsular material derived from the lower organisms. The absence of cellulose in bacteria is of no great import, for many of the true fungi have no cellulose wall and are protected

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[1] We are under obligations to Dr. William H. Park and his associates, of the New York Board of Health, and to Dr. A. P. Hitchens, of Glenolden, Pa., who have kindly placed large amounts of diphtheria and tubercle bacilli at our disposal; and to Professor Hans Zinsser for numerous courtesies.

only by a delicate membrane; unfortunately the evidence as to the presence or absence of chitin, which might have some bearing on the subject, is conflicting.

Bacteria do, however, contain fats and lipoids in varying amounts, which, because of their marked effect on surface tension, would for purely physical reasons tend to become concentrated at the periphery of a colloidal system such as the bacterial protoplasm. With or without a morphologically distinct limiting membrane we can reasonably assume that the external surface of the bacterial cell is potentially lipoidal. Upon such a substrate we should expect lipolytic rather than proteolytic action. Immunologists have recognized that in cytolysis there are other factors than such distinctly protein manifestations of immunity as are exemplified by the precipitin and agglutinin reactions. Zinsser (7) has recently emphasized this fact. In this connection it is interesting to recall again an observation of Metchnikoff (8). Metchnikoff noted that the intestinal tract of the larva of certain insects (moths) was free from bacteria, and reasoned that this absence of intestinal flora must have some connection with the nature of the enzymes secreted into the gastro-intestinal tract. He calls attention to the fact that these insects, utilizing waxes and fats to a large extent, must secrete powerful lipases.

Oppenheimer (9), reviewing the subject of bacteriolysis from an entirely different standpoint, reaches a similar conclusion. He says: "I have formerly classified these substances (cytolysins) among the ferments because the view was prevalent that the destruction of the cell membrane was due to a specific proteolytic ferment." This view must be discarded, but it is probable that we may later place them among the lipases (10). In a recent paper (11) we have shown by microchemical analysis that bacteriolysis is not necessarily associated with proteolysis.

As briefly outlined, the failure of proteolysis by the action of proteolytic ferments on the intact cell offers then no problem in itself, for there is probably no exposed protein substrate upon which the enzyme may act.

Kantorowicz (3) has probably studied the subject most carefully. He confirmed the older observations of the absence of digestion of the intact cell whether living or dead, and showed that heating Gram-negative organisms to 70° C. made them lose their power of resistance, while Gram-positive organisms would resist digestion even after boiling. He concluded that the resistance was due to an antiferment. He found that dried organisms were as resistant as fresh bacteria, but noted that after drying, grinding in a mortar, and extracting with acetone, the organisms lost their resistance. He assumed two factors to be concerned in bacteriolysis, one of which had to do with the overcoming of the antiferment. Kruse (1) did not, however, agree with the conclusion of his pupil, and doubted that an antiferment as such was involved in the resistance of the organisms. He inclined to the view that differences in resistance among bacteria were due to differences in permeability of the organisms.

Fermi (2) working with cultures, introducing ferments into culture media, reached conclusions similar to those of Kantorowicz. He noted, however, that dried organisms were less resistant than those freshly killed.

The foregoing observations were made by using suspensions of organisms either living or killed in various ways, to which the enzyme was added and the resulting digestive effect observed by the relative clearing of the suspensions. This method is open to several objections. The interpretation of the results is inexact and arbitrary, depending on the observer's judgment, and varying under different conditions. Probably of more importance is the fact that solution of the organisms is interpreted as proteolysis. Solution of a bacterial cell may be largely a physical process related most intimately to changes in its lipoidal constituents. The actual proteolytic cleavage is a purely chemical phenomenon and cannot be determined by ocular observation. This distinction, to which Jobling and Strouse (10) have previously called attention, should be clearly kept in mind, for the careless use of these terms has led to considerable confusion in the interpretation of some immunological experiments. There is, furthermore, a technical error in that it is not possible by the means so far employed to present an approximately equal amount of substrate for the enzyme action, which is important when the comparative rate of digestion of different organisms is sought.

In order to obtain results which would be free from the objections above referred to, our observations have been based exclusively on the determination of non-coagulable nitrogen before and after digestion. These were made by means of the Folin method. Only by such a method can we determine the exact percentage of digestion. We have used organisms grown on agar in large flat bottles. The bacteria were washed, dried *in vacuo* at a low temperature, and then ground in an agate mortar. During the process of drying changes must occur in the colloidal state of the external limiting membrane, so that the uniform resistance of various bacteria to proteolytic action while intact is overcome, and the degree of resistance remaining should be an index of the actual antiferment power of the different organisms.

One other physical factor, the intimacy of the lipoid-protein combination, must be taken into consideration. Numerous workers have pointed out that the lipoids occurring in cells are so closely combined with the proteins that it is impossible by the ordinary

extraction methods to break up their union. This can be accomplished only by a complete hydrolysis of the protein. Thus the determination of bacterial lipoids by means of extraction in the Soxhlet apparatus will give results which are much too low, the real ratio of the lipoidal substances being obtained by extraction after saponification. We have noticed during the course of the work that the lipoids from the Gram-positive bacteria are proportionately extracted more slowly by means of lipoid solvents than from the Gram-negative organisms.

The bacterial emulsions were, as a rule, made up in a 1 per cent. suspension. When fresh organisms were used an effort was made to have the bacterial concentration about one milligram of nitrogen per cubic centimeter. The total non-coagulable nitrogen both before and after digestion was determined by precipitating the coagulable protein with acetic acid and salt, boiling for ten minutes, filtering through kaolinized hard filter paper, and making the usual Folin determination on the filtrate. The trypsin used was freshly prepared before each experiment by dissolving a dried preparation in physiological salt solution.

The lipoid content of the bacteria was determined by saponifying on the water-bath for two hours with alcoholic potash, acidifying, and extracting thoroughly with ether. The iodin value of the lipoids was obtained by the usual Wijs method. It should be noted, however, that the iodin determinations, while often repeated, were of necessity carried out on very small amounts of lipoids, and some caution must be observed in basing conclusions on single observations. When it was not possible to repeat the determinations, we have placed a question mark with the figure to denote the fact. Further observations which we hope to make on larger amounts of material will no doubt give more accurate values. It is reasonable to assume that the lipoid extract and the degree of unsaturation will show marked fluctuations under the varying conditions of growth, nutrition, age, and oxygenation, and, as will be noted, the variations in the total lipoid content of different growths of the same organism were at times marked. Lyons (12) has studied the influence of sugars on the fat content of bacteria, and Cramer (13) has carried out similar experiments.

The amount of lipoids (including fats) has been determined by several observers, as shown in the following table (table I). Inasmuch as the majority of these observations were made by simple ether extraction, the values given are in some instances probably much too low.

TABLE I.

Organism.	Lipoids (including fats).	Observer.
Tubercle bacilli	36–44 per cent.	Baudran (14).
Tubercle bacilli	40 per cent.	Kresling (15).
Tubercle bacilli	31.56 per cent.	Levene (16).
Tubercle bacilli	37.57 per cent.	de Schweinitz and Dorset (17).
Tubercle bacilli	26.2–28 per cent.	Hammerschlag (18).
Bacillus mallei	7.91 per cent.	de Schweinitz and Dorset (19).
Diphtheria bacillus	1.62 per cent.	Dzierzgowski and Rekowski (20).
Meningococci	5.94 per cent.	Ditthorn and Woerner (21).
Pneumobacillus	1.7 per cent.	Brieger (22).
Anthrax bacillus	7.8 per cent.	Dyrmont (23).
Bacillus prodigiosus	4.83 per cent.	Kappes (24).
Bacillus xerosis	8.06 per cent.	Kappes (24).

PROTOCOL I.

Effect of Lipoidal Extraction on the Rate of Digestion of Tubercle Bacilli.

Bacterial suspension 1 c.c.	Trypsin.	Total nitrogen.	Gross digestion.	Digestion, per cent.	Lipoid content, per cent.
Tubercle bacilli[2] (dried)	0.2 c.c.	0.55 mg.	0.13	23	31.2
Tubercle bacilli (extracted with ether, chloroform, or alcohol)	0.2 c.c.	0.74 mg.	0.33	44	9
Tubercle bacilli (extracted in Soxhlet apparatus) (ether 120 hrs.; alcohol 100 hrs.; benzol 50 hrs.)	0.2 c.c.	0.80 mg.	0.46	57	7

The suspensions were made up with an equal amount of sodium carbonate solution.

To these we can add the following determinations:

Tubercle bacilli	32.7 per cent.
Staphylococci	4.51–8.5 per cent.
Diphtheria bacilli	5.5 –7.5 per cent.
Typhoid bacilli	7.0 –8.2 per cent.
Colon bacilli	4.2 –8.15 per cent.
Subtilis bacilli	1.7 per cent.

There are no available determinations of the iodin values of

[2] Previously killed by boiling.

these lipoids with the exception of those made upon the waxes and fats of the tubercle bacillus.

The values which we have found are as follows:

Tubercle bacilli	20 for total lipoids; 24.2 for fatty acids.
Staphylococci	60–80
Diphtheria bacilli	80–110
Typhoid bacilli	33–38
Colon bacilli	32–40
Subtilis bacilli	44 (?)
Tetanus bacilli	44 (?)

Having previously shown that the unsaturated fatty acid radical is antitryptic and its inhibition proportional to the degree of unsaturation (25); that such unsaturated lipoids can be isolated from tubercle bacilli (26) and from tuberculous caseous material (27); that serum antitrypsin consists of similar lipoids, either as free fatty acids, as esters of cholesterol, or combined with lecithin (28), we considered it reasonable to assume that bacteria might resist digestion in a degree proportional to the amount of the unsaturated lipoids contained. It will be remembered that Kantorowicz found that the antiferment was removed from the bacteria when they were ground up and extracted with acetone.

INFLUENCE OF LIPOIDAL EXTRACTION ON DIGESTIBILITY.

We first determined the influence of lipoidal extraction on the tryptic digestion of tubercle bacilli. 1 per cent. suspensions of (1) freshly dried organisms, of (2) extracted bacilli (with 9 per cent. of lipoids), and of (3) extracted bacilli (with 7 per cent. of lipoids) were used. The rate of digestion is shown in protocol I. The organisms used in this work were obtained from Dr. Hitchens and had been killed by heating at high temperatures. In addition they were old, and so some of the unsaturated fatty acids may have become oxidized.

A similar result was obtained with other organisms, as is shown in the following experiment with typhoid and colon bacilli (protocol II). Müller (29) noted that typhoid bacilli from which the lipoids had been extracted were more easily digested by leucocytes.

PROTOCOL II.

Effect of Lipoidal Extraction on the Rate of Digestion of Colon and Typhoid Bacilli.

Bacterial suspensions 1 c.c.	Total nitrogen.	Non-coagulable nitrogen.	Substrate.	Gross digestion.	Increase in non-coagulable nitrogen.	Digestion, per cent.
Colon bacilli.....	1.77 mg.	0.17 mg.	1.60 mg.	0.545 (−0.17)	0.375 mg.	23
Ether-extracted colon bacilli.....	1.87 mg.	0.23 mg.	1.64 mg.	0.95 (−0.23)	0.72 mg.	44
Typhoid baciili.....	1.08 mg.	0.14 mg.	0.94 mg.	0.66 (−0.14)	0.52 mg.	56
Ether-extracted typhoid bacilli.....	1.0 mg.	0.2 mg.	0.8 mg.	0.87 (−0.2)	0.67 mg.	84

THE LIPOIDS AS ANTIFERMENTS.

We have previously noted that the lipoids extracted from tubercle bacilli were antitryptic when saponified (26). That the same is true of the lipoids from other organisms is shown in the following experiment.

Pure cultures of staphylococci and of colon bacilli were removed from bottles, washed, and saponified. After acidification, the lipoids were extracted and again saponified. The soaps were then dissolved in physiological salt solution. Various amounts were mixed with one unit of trypsin, incubated for thirty minutes, and casein was then added for digestion. In another set of tubes the unsaponified bacterial lipoids, suspended in salt solution, were used in equal amounts. The relative inhibition will be noted in protocol III.

PROTOCOL III.

Effect of Bacterial Lipoids, Saponified and Emulsified, on Tryptic Digestion.

Tube No.	Casein.	Trypsin.	Staphylococci lipoids saponified.	Colon lipoids saponified.	Staphylococci lipoids emulsified.	Colon lipoids emulsified.	Gross casein digestion.	Digestion, per cent.
1	2 c.c.	0	0	0	0	0	0.0 mg.	0
2	2 c.c.	0.1 c.c.	0	0	0	0	1.66 mg.	100
3	2 c.c.	0.1 c.c.	2 c.c.	0	0	0	0.217 mg.	13
4	2 c.c.	0.1 c.c.	0	2 c.c.	0	0	0.715 mg.	43
5	2 c.c.	0.1 c.c.	0	0	2 c.c.	0	1.33 mg.	80
6	2 c.c.	0.1 c.c.	0	0	0	2 c.c.	1.54 mg.	92

The iodin value of the lipoids was 97 for those from the staphylococci, and 32 from the colon bacilli.

THE COMPARATIVE RATE OF DIGESTION.

We next studied the comparative rate of digestion of bacteria, and the relation to the lipoids contained in the bacteria. Such an experiment is shown in protocol IV, and has for convenience been charted (text-figure 1). As a lipoidal antitryptic index we may multiply the percentage of lipoids by the iodin value, as determined for the organisms used in this experiment.

PROTOCOL IV.

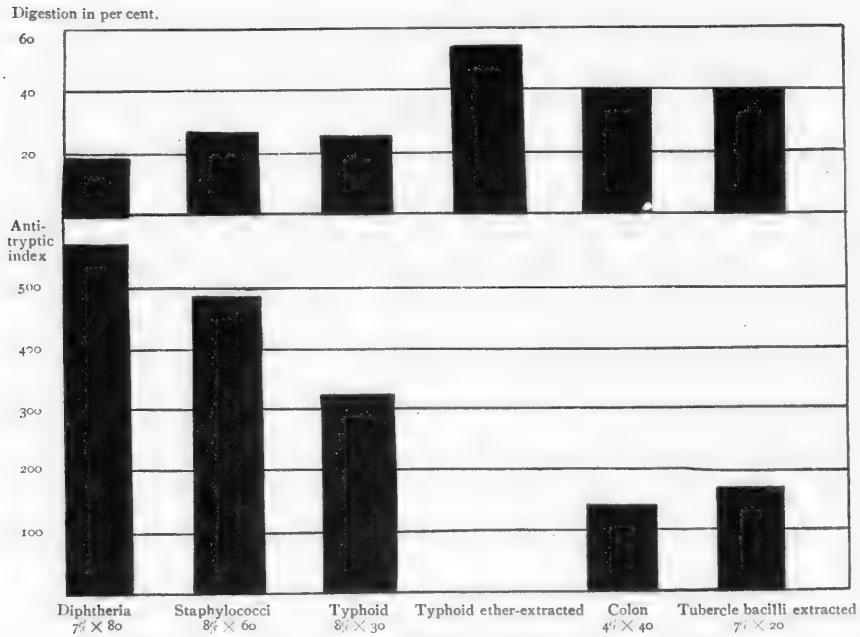
Comparative Rate of Tryptic Digestion of Bacteria.

Bacterial suspension 1 c.c.	Total nitrogen.	Non-coagulable nitrogen.	Substrate.	Gross digestion.	Increase in non-coagulable nitrogen.	Digestion, per cent.
Diphtheria bacilli	1.0 mg.	0.12 mg.	0.88 mg.	0.28(−0.12)	0.16 mg.	18
Staphylococci	0.83 mg.	0.15 mg.	0.68 mg.	0.31(−0.15)	0.16 mg.	23
Typhoid bacilli	1.42 mg.	0.21 mg.	1.21 mg.	0.5 (−0.21)	0.29 mg.	24
Typhoid bacilli (ether-extracted)	0.9 mg.	0.35 mg.	0.55 mg.	0.66(−0.35)	0.31 mg.	56
Tubercle bacilli (ether-extracted)	0.83 mg.	0.12 mg.	0.71 mg.	0.4 (−0.12)	0.28 mg.	40
Colon bacilli	0.8 mg.	0.12 mg.	0.68 mg.	0.39(−0.12)	0.27 mg.	40

Diphtheria bacilli ..... 7 per cent. × 80 = 560
Staphylococci ..... 8 per cent. × 60 = 480
Typhoid bacilli ..... 8 per cent. × 30 = 240
Typhoid bacilli (extracted) ..... ? ? ?
Colon bacilli ..... 4 per cent. × 40 = 160
Tubercle bacilli (extracted) ..... 7 per cent. × 20 = 140

This offers, of course, only a general approximation, and cannot take into consideration the fact that the lipoids vary no doubt in the intimacy of their connection with the proteins, a factor which must influence their ability to protect the protein. The digestion as determined was as follows:

Diphtheria bacilli ..... 18 per cent.
Staphylococci ..... 23 per cent.
Typhoid bacilli ..... 24 per cent.
Typhoid bacilli (extracted) ..... 56 per cent.
Colon bacilli ..... 40 per cent.
Tubercle bacilli (extracted) ..... 40 per cent.



TEXT-FIG. 1. Relation of the antiferment index to the rate of digestion of various organisms.

These figures show a striking parallelism between the antitryptic property and the lipoidal protecting bodies.

A similar experiment can be shown if we compare the rate of digestion of the Gram-positive organisms, the lipoids being as follows:

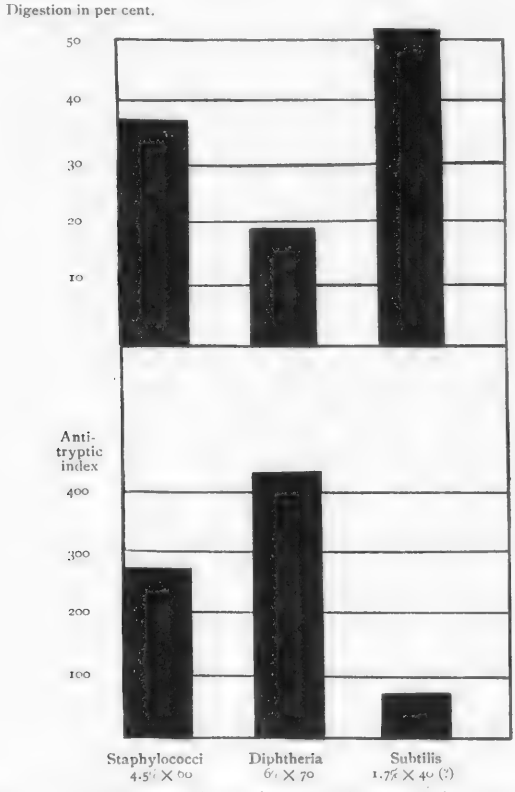
Diphtheria bacilli ..... 6.0 per cent. × 70 = 420
Staphylococci ..... 4.5 per cent. × 60 = 270
Subtilis bacilli ..... 1.7 per cent. × 40 = 68

PROTOCOL V.

Comparative Rate of Digestion of Staphylococci and Diphtheria and Subtilis Bacilli.

Bacterial suspension 2 c.c.	Total nitrogen.	Non-coagulable nitrogen.	Substrate.	Gross digestion.	Increase in non-coagulable nitrogen.	Digestion, per cent.
Staphylococci.....	1.54 mg.	0.375 mg.	1.17 mg.	0.8 (−0.375)	0.425	36
Diphtheria bacilli.....	1.66 mg.	0.19 mg.	1.47 mg.	0.47 (−0.19)	0.27	19
Subtilis bacilli.....	1.3 mg.	0.48 mg.	0.82 mg.	0.909 (−0.48)	0.429	52

The organisms digested in the order indicated, the digestion being least with the diphtheria bacilli. The results are shown in protocol V and in text-figure 2.



TEXT-FIG. 2. Relation of the antiferment index to the rate of digestion of Gram-positive organisms.

THE EFFECT OF HEAT.

It has been mentioned that a difference existed in the resistance to heat of the Gram-positive and the Gram-negative organisms. This is shown in the following experiments.

A suspension of typhoid bacilli was heated at 50°, 60°, 70°, and 100° C. The

tubes were then subjected to the action of trypsin. The percentage of digestion was as follows:

50° C. = 31 per cent.
60° C. = 31 per cent.
70° C. = 36 per cent.
100° C. = 41 per cent.

As Kantorowicz (3) has found, there is a reduction of the resistance after heating to 70° C., but the effect is not so striking as one might expect if a complete destruction of the antiferment had resulted. The Gram-positive organisms, staphylococci for example, show no change after heating, unless it is a slight increase in the resistance. The percentage of digestion was as follows:

Unheated = 55 per cent.
50° C. = 54 per cent.
60° C. = 35 per cent.
70° C. = 41 per cent.
100° C. = 38 per cent.

In a second experiment the results were as follows:

Unheated organisms = 16 per cent.
70° C. = 18 per cent.
100° C. = 14 per cent.

LIPOIDAL ADSORPTION BY BACTERIA.

We have previously mentioned that bacteria suspended in normal serum are able to adsorb the unsaturated lipoids from the serum, and by virtue thereof to become more resistant to the action of trypsin (30). Such an experiment follows.

A suspension of typhoid bacilli was incubated with 10 c.c. of normal guinea pig serum for six hours. The bacteria were then centrifuged, washed, and dried at a low temperature. Untreated bacteria were used as a control. Emulsions were prepared containing 1 mg. of nitrogen per cubic centimeter, and to each was added 0.1 c.c. of trypsin solution and 2 c.c. of a 0.5 per cent. solution of sodium carbonate. Digestion was permitted for six hours. The digestion of the untreated bacteria was 75 per cent., of the serum-treated bacteria 23 per cent.

The lipoidal adsorption can be shown in a more direct manner, as is illustrated in the following experiment.

Dried subtilis bacilli were added to 25 c.c. of horse serum and incubated for three hours. The bacteria were then washed after being centrifuged from the serum. The lipoid content of the bacteria and of the supernatant serum was now determined.

Total lipoids in 25 c.c. of original serum	0.083	gm.
Total lipoids in 25 c.c. after incubating with bacteria	0.060	gm.
	0.023	gm. loss.
Weight of bacteria recovered	0.6	gm.
Lipoid content originally present, 1.7 per cent.	0.0102	gm.
Total lipoids recovered from bacteria	0.036	gm.
Less lipoids originally present	0.0102	gm.
	0.0258	gm. gain.

While there has been a loss of 0.023 of a gram in the ether-soluble substances from the serum, the bacteria show a corresponding gain. The absorption of lipoids is made especially evident in this case because of the small lipoid content of the organisms used. The lipoid determinations were made after thorough saponification of both bacteria and serum on the water-bath for three hours.

DISCUSSION.

Inasmuch as the bacterial lipoids may show more marked fluctuations than the other constituents, the quantity depending largely on the composition of the nutrient media and to some extent on the age of the culture, and the chemical quality, unsaturation, being subject to changing oxidative conditions, we are of the opinion that the demonstration that the antiferment property of bacteria depends on definite quantitative as well as qualitative differences in the lipoidal constituents of the bacteria may offer the means of solution of some still obscure problems in immunity. Thus in the study of virulence, depending on the invasive power of the organisms and on their resistance against the defensive mechanism of the host a partial solution might be found in a comparative study of the lipoids under different conditions of bacterial existence. We have experiments under way to elucidate this point. So too, a further study of the adsorption of the lipoids from the serum offers an attractive field. While in this process the resistance of the intact bacteria to proteolytic enzymes is not increased, for on such organisms the

enzymes probably find no substrate upon which they may become fixed and active, nevertheless the metabolic processes of the cell, depending wholly upon the rate of exchange of nutritive and excretory products through the cell membrane, must be profoundly altered. Indeed it might be conceived that such a disturbance of the proper functioning of the membrane would under some conditions lead to the death of the cell. On the other hand, an absorption of serum lipoids, by preventing the excretion of toxic metabolic products, such as leucocydins, virulins, etc., might bring about phagocytosis of organisms otherwise resistant. That the so called opsonins are closely related to the lipoids seems to have been established by the work of Stuber (31).

That the bacterial lipoids influence the intracellular proteolytic activity becomes apparent when we compare the amount of non-coagulable nitrogen contained in various organisms. Thus diphtheria bacilli, with a high antiferment index, have only from 5 to 7 per cent. of the nitrogenous material in a non-coagulable form, whereas in subtilis bacilli, with a low antiferment index, a correspondingly greater protease activity is indicated by the presence of from 33 to 35 per cent. of the total nitrogen in a non-coagulable form. Staphylococci, typhoid bacilli, and colon bacilli occupy an intermediate position. This influence is analogous to the effect of the antiferment on the protein metabolism of higher organisms, as we shall show in a subsequent paper.

The antiferment, being non-specific, is able not only to inhibit the action of the digestive ferments, but is probably the agent concerned in preventing excessive protease action, or autolysis, in the cells. It is easy to understand that with the death of the cells and subsequent development of an acidity because of the failure in the removal of acid waste products, the antiferment property is greatly lessened by changes in the state of dispersion brought about by the increase in acidity. This lowering of the antiferment property of the unsaturated lipoids by a change in their dispersion is most easily demonstrated with serum antitrypsin. The morphological expression of the process is possibly to be found in the development of the so called myelin figures found in cells stained during the early stages of autolysis. It is probable that the increase in the rate of

autolysis following the addition of certain inorganic colloids to liver emulsions, noted by Ascoli and Izar (32), depends on an absorption of the lipoidal antiferments.

Kantorowicz (3), in view of the difference in behavior of the antiferment in Gram-negative and Gram-positive organisms, is inclined to the view that the antiferments are in this case different. It seems more reasonable to assume that the difference noted is due to a purely physical condition. It is possible that in the Gram-positive organisms the lipoids are in more intimate association with the protein molecule, so that when dispersion changes are brought about, as by heating, the relative protection by these lipoids remained unaltered. This idea would find support in view of the fact that the lipoids from Gram-positive organisms resist extraction by lipoid solvents to a greater extent than do those from Gram-negative bacteria.

In view of the work of Tamura (33), definitely showing that the bacterial constituent responsible for the Gram stain is lipoidal in that it can be found in the lipoid extract, and as the unsaturated fatty acids and their salts take the Gram stain easily, because the iodin can be bound to the unsaturated carbon atoms, it would seem rational to seek some connection between the degree of unsaturation of the bacterial lipoids and the Gram staining ability. From this point of view the high iodin values of the lipoids from diphtheria bacilli and staphylococci, as compared with those of typhoid or colon bacilli, might seem significant, were it not for the fact that in the case of the tubercle and subtilis bacilli, the results are apparently the reverse. Knudson (34), working in Gies's laboratory, finds, however, that the lipoids from tubercle bacilli, which have a low iodin value (24), contain several of the higher unsaturated fatty acids.

As a result of our study we are inclined to emphasize the importance of the unsaturated lipoids as representing probably a most important factor in the regulation of the proteolytic activity of the cellular metabolism, due to the fact that the protein lipoid combination resists the action of the proteolytic enzymes.

CONCLUSIONS.

1. Intact bacteria probably resist tryptic digestion because of the absence of an exposed protein substrate.

2. Dried organisms resist digestion in a degree proportional to their content of unsaturated lipoids.

3. Lipoidal extractives reduce the resistance to tryptic digestion.

4. The extracted lipoids (saponified) are antitryptic in a degree proportional to their unsaturation.

5. The inactivation of the antiferment in Gram-negative organisms is probably due to changes in the degree of lipoidal dispersion.

6. Bacteria adsorb lipoids from the serum when incubated at 37° C. Such organisms when dried are found to be more resistant to tryptic digestion than untreated organisms.

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LIPOIDS AS INHIBITORS OF ANAPHYLACTIC SHOCK.

Studies on Ferment Action. XVIII.*

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The refractory period following anaphylactic shock, noted early in the study of anaphylaxis, has been variously explained. Among immunologists the idea seems to be generally accepted that the condition is due to an exhaustion of the specific immune substance which together with complement is supposed to act upon the introduced antigen.

Rusznjak (1) first noted that immediately following anaphylactic shock, especially if protracted or following an extended latent period, a definite rise in the antitryptic titer of the animal's serum occurred. Rusznjak, assuming that the hypothesis of Rosenthal (2) in regard to the nature of serum antitrypsin was correct, interpreted his experiment as a demonstration of the splitting of proteins during anaphylactic shock. He furthermore advanced the idea that the period of resistance following shock was due to this increase in the antiferment property of the blood. Seligmann (3) could not confirm these findings, but Pfeiffer and Jarisch (4) later found the observations to be correct, and furthermore showed that a similar rise in antitrypsin occurred following various protein intoxications,—hemolysins, protein split products, etc. Zinsser (5) has recently shown that an increased resistance is found to anaphylatoxins following a first sublethal injection, and we have noted a similar condition with serotoxin (6). Following a sublethal serotoxin injection a well marked rise in the antitryptic titer is observed.

The observation of Rusznjak is therefore true not only for anaphylaxis but probably for every intoxication accompanied by cellular destruction. His conclusions, however, being based on the erroneous theory of Rosenthal as to the nature of serum antitrypsin, are incorrect. We have recently demonstrated that the unsaturated lipoids of the serum are the substances upon which the antitryptic property depends (7). The increase in antitrypsin observed in these cases cannot be due directly to the protein split products, but is to be explained by

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the liberation of lipoids following cellular destruction. The observations of Rusznjak and of Pfeiffer and Jarisch offer then no direct evidence that protein splitting occurs during anaphylactic shock, but merely indicate that a general cellular intoxication has occurred.

The increase in antiferment might nevertheless explain the increased resistance to a second injection of antigen in a sensitized animal. This question can find a solution only if it is possible to increase the antiferment in the blood serum by some means other than a protein intoxication, for following such injury the objection can be made that there is a reduction or exhaustion of both specific and non-specific proteases or antibodies responsible for the shock, together with a destruction of complement. That complement, however, can have no relation to the phenomena, if we regard the intoxication as purely protein, is evident from the fact that the complementary action is not proteolytic but probably lipolytic, a subject which we have briefly discussed in a previous paper (8). Serum antitrypsin being lipoidal and capable of isolation from the serum by means of lipoidal extractives should on reinjection into experimental animals cause an increase in the antiferment property of the blood serum. Such an experiment can be made as follows.

METHODS OF INCREASING THE ANTIFERMENT IN THE BLOOD.

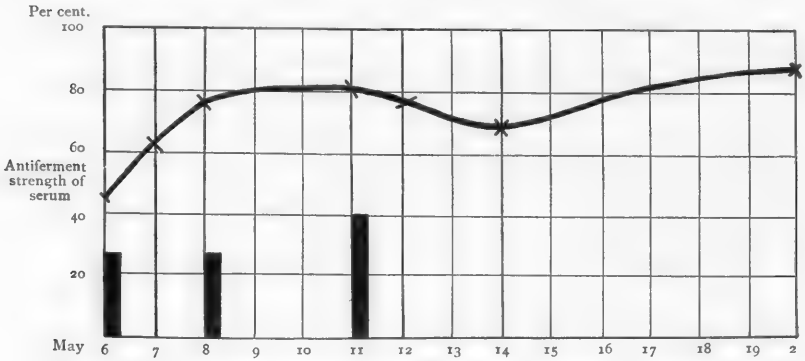
About 300 milligrams of serum lipoids extracted at various times from human and guinea pig serum were dissolved in six cubic centimeters of olive oil. Of this solution a large guinea pig received subcutaneous injections as follows:

May 6, 1914. 1.5 c.c.
May 8, 1914. 1.5 c.c.
May 11, 1914. 2.0 c.c.

The antiferment index, determined by the method discussed in our former papers, is shown in text-figure 1, in which the inhibition of tryptic digestion by 0.05 of a cubic centimeter of serum both before and after the injections is outlined. It will be noted that there is a distinct and prolonged rise from the original 45 per cent. inhibition to more than 80 per cent. as determined on May 11.

Olive oil injected subcutaneously in equal amounts had no effect on the antitryptic index.

Inasmuch as it is difficult to secure large amounts of serum lipoids we have substituted the fats prepared from egg yolk which contain large quantities of unsaturated lipoids. These were obtained as follows: The yolk was extracted twice with alcohol and twice with ether. The extracts were combined and evaporated to dryness. The mass was then extracted with ether and precipitated with acetone. Only the acetone-soluble lipoids were used. As so obtained the lipoids are semifluid and can be injected without the



TEXT-FIG. 1. Increase in antitrypsin following injection of serum lipoids subcutaneously.

use of a solvent. The increase in antitrypsin following the subcutaneous injection of one cubic centimeter in a guinea pig is shown as follows:

	Inhibition by serum before injection. June 29, 1914.	After injection. July 3, 1914.
0.10 c.c. serum	69 per cent. inhibition	79 per cent.
0.075 c.c. serum	44 per cent. inhibition	70 per cent.
0.05 c.c. serum	10 per cent. inhibition	70 per cent.
0.025 c.c. serum	0 per cent. inhibition	0 per cent.

The guinea pig was of medium weight. It was bled from the heart (two cubic centimeters) on June 29, and the egg fat injected the same day. The rise in antiferment is marked.

THE INHIBITION OF ANAPHYLACTIC SHOCK.

Having established the fact that these lipoids will, when subcutaneously absorbed, cause an increase in antiferment, we investigated the effect of such injections on the reaction of sensitized guinea pigs when the specific antigen was injected intravenously.

Six guinea pigs were sensitized on May 16, 1914, with 0.1 c.c. of horse serum intraperitoneally. Three of the animals received subcutaneous injections of serum antitrypsin dissolved in olive oil. The injections, 1 c.c. each, were made on May 21, May 26, and June 5. Purified horse serum albumen solution (1 per cent.) was used for reinjection. The toxic dose of this was determined on the three control animals and resulted as follows:

Animal No.	Weight	Dose.	Dose per gm. weight.	Result.
1	240 gm.	0.8 c.c.	0.0033 c.c.	Convulsions, respiratory spasms. Final recovery in 10 min.
2	280 gm.	1.4 c.c.	0.005 c.c.	Death in 2 min. Typical.
3	300 gm.	1.5 c.c.	0.005 c.c.	Same.

The lethal dose, 0.005 c.c. per gram weight, was now injected into the antitrypsin guinea pigs.

Animal No.	Weight.	Dose.	Dose per gm. weight.	Remarks.
4	210 gm.	1.0 c.c.	0.0047 c.c.	Scratches. No convulsions, no respiratory spasms. Remained well.
5	300 gm.	1.5 c.c.	0.005 c.c.	Death in 3 min. Typical.
6	220 gm.	1.1 c.c.	0.005 c.c.	No symptoms except restlessness. Remained well.

In two of these guinea pigs the acute shock had been completely averted. A similar and even more striking effect was obtained with guinea pigs treated with egg fats.

Ten guinea pigs were sensitized with horse serum on July 1, 1914. Six of the animals were injected previously with 1 c.c. of egg fat, on June 29. One of the animals was killed on July 3 and the antitryptic index determined, the result being shown on page 470. They received further injections of 1 c.c. each on July 10 and 16. The effect of the reinjection of a 1 per cent. horse serum albumen solution is shown in the following table.

CONTROL ANIMALS.

Animal No.	Weight.	Dose.	Dose per gm. weight.	Result.
1	170 gm.	0.425 c.c.	0.0025 c.c.	No effect.
2	230 gm.	1.15 c.c.	0.005 c.c.	Marked respiratory convulsions and spasms. Recovered in 30 min.
3	295 gm.	2.95 c.c.	0.01 c.c.	Death in 2 min. Typical.
4	275 gm.	2.75 c.c.	0.01 c.c.	Death in 2 min. Typical.

The minimum lethal dose was therefore 0.01 of a cubic centimeter per gram weight of guinea pig. The injection into the animals treated with egg fat resulted as follows:

Animal No.	Weight.	Dose.	Dose per gm. weight.	Result.
5	360 gm.	3.6 c.c.	0.01 c.c.	Immediate respiratory spasms; gradual recovery. Well after 10 min.
6	290 gm.	2.9 c.c.	0.01 c.c.	Scratched; no other symptoms.
7	235 gm.	2.35 c.c.	0.01 c.c.	No symptoms.
8	190 gm.	2.37 c.c.	0.0125 c.c.	No symptoms.
9	240 gm.	4.8 c.c.	0.02 c.c.	Marked respiratory spasms, with dyspnea for 20 min. Complete recovery.

It will be observed that all the animals receiving from one to two times the lethal dose made a complete recovery, and in three of the guinea pigs no symptoms of note were observed. The animals remained well during the next two weeks while under observation. There can then be no question but that the increase in antiferment is able to protect the animal from at least twice the minimum lethal dose of antigen and that this increase in antiferment following a protein shock must have a large share in the resistance to a second injection.

THE INFLUENCE OF LIPOIDS PRESENT IN THE ANTIGEN ON ANAPHYLACTIC SHOCK.

It has been found difficult to induce acute anaphylactic shock by means of whole bacteria, and differences have been noted in the period of time following a second injection of various antigens before symptoms of shock would be elicited, the latent period varying from one to fifteen minutes. A long latent period is common with egg albumen; indeed, Rusznjak employed egg albumen for this reason in his experiments. Inasmuch as we have recently demonstrated (9) that bacteria contain unsaturated lipoids which represent the antiferment and that they resist digestion in a degree proportional to the amount of the lipoids present; and since egg albumen resists digestion by means of the ordinary tryptic ferment unless first acted upon by pepsin in an acid medium, whereby the antiferment is destroyed because of alteration in the colloidal dis-

persion brought about by the change in reaction, we next examined the relative toxicity of an antigen before and after chloroform extraction. For this purpose we used the same preparation of purified horse serum albumen, the method of preparation of which we have discussed previously (6).

Fifty cubic centimeters of a 1 per cent. solution were prepared, of which twenty-five cubic centimeters were thoroughly shaken with chloroform and incubated for forty-eight hours, the flask being shaken at intervals. No autolysis occurred during this time. Before use the chloroform was freed from the serum albumen solution by centrifugation and filtration through a coarse paper filter. Guinea pigs were sensitized with horse serum intraperitoneally on April 13, 1914. The minimum lethal dose of the original horse serum albumen solution was determined as follows:

May 20, 1914.

Animal No.	Weight.	Dose.	Dose per gm. weight.	Result.
1	300 gm.	3.0 c.c.	0.01 c.c.	Typical anaphylactic shock. Death in 2 min.
2	300 gm.	1.5 c.c.	0.005 c.c.	Same.
3	360 gm.	0.9 c.c.	0.0025 c.c.	Recovered.
4	310 gm.	0.77 c.c.	0.0025 c.c.	Typical death in 3 min.
5	270 gm.	0.32 c.c.	0.0012 c.c.	Scratched; no other symptoms.

The minimum lethal dose was therefore about 0.0025 of a cubic centimeter per gram weight. The extracted serum albumen solution was toxic in a much smaller dose, as will be seen from the following table.

Animal No	Weight.	Dose.	Dose per gm. weight.	Result.
6	290 gm.	0.72 c.c.	0.0025 c.c.	Death immediate and typical.
7	290 gm.	0.36 c.c.	0.0012 c.c.	Death immediate and typical.
8	320 gm.	0.2 c.c.	0.0006 c.c.	Death immediate and typical.
9	300 gm.	0.1 c.c.	0.0003 c.c.	Marked convulsions; respiratory spasms. Final recovery.

This experiment would indicate that the lipoids combined with the protein antigen may exert a considerable influence on the relative toxicity of the anaphylactic antigen, and probably explains

the difficulty encountered in obtaining uniform results with such antigens as bacteria which are relatively well protected by their lipoidal components.

THE PREVENTION OF ANAPHYLACTIC SHOCK BY THE SIMULTANEOUS INJECTION OF SOAP SOLUTIONS.

If by extraction of a lipoid containing protein antigen it becomes more toxic, it would seem reasonable that by adding the extracted lipoids to the same the toxicity should be decreased, or, if added in sufficient amount, completely neutralized. We have shown that this can actually be done with serotoxin (6), and we discussed the reasons for the use of soaps in place of lipoid suspensions. A difficulty is encountered, though, in the use of soaps, in that they will in themselves on injection cause a shock similar to that observed in anaphylaxis, so that great care must be used in working with doses that are sublethal (10). While such soaps, oleates for example, are highly antitryptic, we have noted (7) that when incubated with serum, instead of increasing the antiferment index as might be expected, they actually cause a lowering of the antitryptic titer, possibly because of solution of the serum antiferment in the soap solution. Nevertheless, when added to the anaphylactic antigen, soap solutions are able to render the reinjection harmless within certain limits, as will be observed in the following protocols. We may state, however, that we have not been able to secure these results when whole serum was used as an antigen; the results have been obtained only when a solution of horse serum albumen was used. Whether this depends on the greater stability of the albumens as contrasted with the globulins, as might be indicated in the recent work of de Waele (11), or whether it is due to the fact that the soaps lower the antiferment index when added to the whole serum, in which case the injected antigen would have less protection than before, we cannot state.

Sensitized animals and antigen solutions, the toxic doses of which have been described on page 473, were used. 1.5 cubic centimeters of the original serum albumen solution were mixed with one cubic centimeter of a 1 per cent. solution of sodium oleate and incubated

for ten minutes. The surely toxic dose of the antigen was 0.005 of a cubic centimeter per gram weight.

Weight of animal.	Dose.	Dose per gm. weight.	Result.
300 gm.	1.5 c.c. 1.0 c.c. soap solution	0.005 c.c.	No symptoms.

Similar mixtures were made with the lipoid-extracted serum albumen solution, the lethal dose of which was 0.0006 of a cubic centimeter per gram weight.

Weight of animal.	Dose.	Dose per gm. weight.	Result.
280 gm.	0.2 c.c. 1.0 c.c. soap solution	0.0007 c.c.	No effect.
260 gm.	0.4 c.c. 1.0 c.c. soap solution	0.0015 c.c.	No effect.
260 gm.	1.0 c.c. 1.0 c.c. soap solution	0.0038 c.c.	No effect.

These experiments are simply isolated examples from numerous trials which have always shown the same result. In the last protocol the protection is observed to be ample against five times the minimum lethal dose.

Whether or not this protection by the soaps is a mechanical one, due to the formation of a thin soap membrane about the aggregates of the antigen, whether it is due to the actual antiferment property of the soap, or whether its action depends on changes induced in the cellular membranes of the animal so injected, rendering the cells less permeable to the toxic substances responsible for the symptom-complex, can, of course, not be decided from these experiments. We are, however, inclined to assume that the latter is the explanation, especially in view of the work of Schultz (12), Dale (13), Weil (14), and Coca (15), showing that the origin of the shock is probably cellular and not humoral.

CONCLUSIONS.

1. The antitryptic titer of the serum can be increased by subcutaneous injections of serum lipoids (antitrypsin) and of the lipoids from egg yolk.

2. Animals so injected show a relative immunity to acute anaphylactic shock (two minimum lethal doses).

3. Extraction of lipoids contained in antigens increases the toxicity of the antigen when injected into a sensitized animal.

4. Sublethal doses of soap solutions injected simultaneously with the antigen (purified horse serum albumen) prevent anaphylactic shock.

5. The refractory state following anaphylactic shock is related in part to an increase in the antitryptic titer of the serum.

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FURTHER EXPERIMENTS UPON THE EFFECTS OF EXTIRPATION OF THE THYMUS IN RATS, WITH SPECIAL REFERENCE TO THE ALLEGED PRODUCTION OF RACHITIC LESIONS.*

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PLATES 22 TO 25.

In a previous paper (1) dealing with the effects of extirpation of the thymus of white rats, the writer reached the conclusion that total removal of the gland at an early age was not necessarily followed by modifications of the normal development, nor by characteristic changes in the skeletal system.

While this article was in press, there appeared a short paper by Klose (2) embodying results at variance with these observations. Klose described briefly the results of thymus extirpations in pigs, goats, rats, and chickens. The rats were operated upon on the fourteenth day after birth. Some of the animals showed no results from the operation, and this failure is ascribed by Klose to the presence of accessory tissue within the thyroid gland. In rats without such accessory thymus tissue, death followed after eight to ten weeks, with symptoms of cachexia thymopriva. The bones in these rats showed severe rachitic changes. Most striking were the changes in the ribs, in which there was found a typical rosary, such as is seen in human rickets. Microscopically there was an extreme widening of the zone of growing cartilage, failure of calcification in the preparatory zone, great irregularity and distortion of the cartilage columns, and their penetration by blood vessels. Klose pictures further a dense spongiosa composed in the region of the costochondral junction almost wholly of osteoid, and an accompanying transformation of the marrow into "*Fasermark.*" Similar but less striking changes were found also in the femora.

Magnini (3) has also published an article dealing with the effect of thymus extirpation upon white rats. Young rats, less than 60 or 65 gm. in weight, showed effects noticeable within a few days after the operation. There developed a progressive cachexia which terminated fatally after forty to fifty days. Older rats, up to 70 gm. in weight, survived for about two months, whereas still larger animals recovered and remained alive. By the injection of suitable doses

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of thymus extract, it was possible to prevent the ill effects of the thymus extirpation. The spleen was found enlarged in 75 per cent. of the thymectomized rats, but no microscopic changes were observed in liver, adrenals, hypophysis, or testicles. No mention is made of alterations in the skeletal system.

In view of such conflicting observations, it seems desirable to bring forward additional evidence in support of the conclusions reached in the writer's previous paper. It has been suggested[1] that negative results might be due to unrecognized lobules of accessory thymic tissue which had undergone delymphoidization or other involutional change making their identification difficult. While it is hard to refute this criticism absolutely, it may be said that in those cases in which accessory tissue has been present, there has been no difficulty in recognizing it as such and in differentiating it from lymphoid tissue. Further, the failure of such minute thymic rests to undergo hyperplasia in itself suggests that they are of no great functional significance.

As regards the alleged production of rachitic changes, it was pointed out that Klose, although aware of the occurrence of spontaneous rachitis in rats, had given no data by which one could exclude this disease as a possible explanation of his findings. At that time I had not met with rachitic lesions in rats. During the past spring, however, a number of rats have been studied which showed striking changes in the bones and teeth. Among these were rats which had been thymectomized at an early age, control rats of the same litters, and rats from the stock cages. It is believed that a detailed description of some of these animals will support the view that these alterations of the skeletal system are not due to the lack of thymus secretion, but are the result of the spontaneous disease first described by Morpurgo.

This paper, therefore, includes observations (1) upon litters in which complete thymectomy was not followed by alterations in growth, nutrition, or skeletal system; (2) upon litters in which both operated animals and controls showed lesions of the bones and teeth; and (3) upon unoperated animals from the stock cages, showing similar lesions. Through the coöperation of Professor W. J. Gies, calcium determinations of the bones and teeth were made in

[1] Personal communication from Professor J. Salkind.

a number of the rats, and the results of these determinations are published in a separate paper (4).

The operative technique has been fully described in the previous study. The completeness of the operation and the possible presence of accessory thymic tissue in the thymectomized rats was controlled in each case by an examination of serial sections of the entire neck organs, including the thyroid, and the upper half of the thoracic organs. The teeth and bones were decalcified in 5 per cent. nitric acid. The staining methods included Morpurgo's modification of Schmorl's thionin-picric acid method, which differentiates even in completely decalcified specimens between osteoid and completely calcified bone.

PROTOCOLS.

Litter U.—This litter consisted of seven rats, four of which were thymectomized on July 28, 1913. During the following weeks mother and young received protective inoculations of a *B. enteritidis* vaccine, since fatal infections with this organism were prevalent at that time.

Rat U2.—Emaciated, cyanotic, and anemic; died eight weeks after operation, having gained only 10 gm. It was eaten by the other rats and could not be examined.

Rat U7.—Control. Gained poorly, remained weak, and had a bloody discharge about the eyes and nose. It was killed sixty-eight days after operation. Weight at death 38 gm. No gross or microscopic lesions were found to explain the inanition.

Rat U4.—Thymectomy. This rat also failed to gain weight normally, but remained lively, and aside from poor nutrition, showed no evidences of illness. It was killed sixty-eight days after operation, rat U7 serving as control. Weight at death 34 gm.

Autopsy.—Negative, aside from emaciation.

Microscopic Examination.—Numerous necroses, old and recent, in the liver. There was absence of spermatogenesis. Serial sections of the neck organs showed three lobules of thymic tissue lying in front of the trachea. There was also a small accessory thymus lobule, below and external to the lower pole of the left thyroid lobe.

The remaining rats of the litter (U1, U3, U5, and U6) were kept under observation until January 28 and 29, 1914, when they were killed with ether.

Rat U1.—Thymectomy. On Dec. 6 a small piece of thymus from a rat of another litter (Z4) was implanted in the peritoneal cavity. No symptoms followed this operation, and no vestiges of the implanted fragment were found at autopsy.

Autopsy.—No gross visceral or skeletal changes.

Microscopic Examination.—A few small necrotic areas are seen in the liver,

with accumulations of wandering cells in the portal spaces. Splenic follicles are large, with a broad zone of paler mononuclear cells at the periphery. There are myeloid foci in the pulp, and numerous megakaryocytes. There is also one small collection of polynuclears. Near a lymph node in the region of the salivary gland, there is a small lobule of tissue, in part necrotic, in part composed of pale epithelioid cells. This can not be identified as thymus. Adherent to the pericardium there is a lobule of tissue measuring 500 by 150 by 160 microns, which is composed of oval or fusiform cells with pale nuclei, arranged about capillaries or irregularly distributed and infiltrated with lymphoid and plasma cells. It can not be identified as thymus tissue, since it shows no Hassall bodies nor larger epithelial cell complexes, and is probably granulation tissue.

Rat U3.—Thymectomy.

Autopsy.—No gross lesions in the viscera or skeletal system, save that there are several parasitic (?) cysts of the mesentery.

Microscopic Examination.—There are no infective lesions of the liver or spleen. The series of the neck organs is incomplete in that the upper and posterior portions are torn in places. The mediastinal structures are, however, intact. No thymus tissue is present, and no accessory glands are found.

Rat U5.—Control. There are suppurative foci in the middle lobe of the right lung. The spleen is enlarged. There are no other gross lesions.

Microscopic Examination.—No infective lesions in liver or spleen.

Rat U6.—Control.

Autopsy and Microscopic Examination.—Normal organs. There is active spermatogenesis.

The weights of the viscera and other data are summarized in the following table.

TABLE I.

Rat.	Duration of experiment.	Weight at operation.	Weight at death.	Length.	Weight of spleen.	Weight of testes.	Spermatogenesis.	Weight of adrenals.	Weight of thymus.	Remarks.
U1	185 dys.	14 gm.	97 gm.	151 mm.	0.400 gm.	♀	—	0.025 gm.	—	Complete thymectomy. Infective lesions.
U3	186 dys.	14 gm.	111 gm.	—	0.318 gm.	♀	—	0.030 gm.	—	Complete thymectomy.
U4	68 dys.	14 gm.	36 gm.	124 mm.	0.220 gm.	0.180 gm.	0	0.020 gm.	—	Incomplete thymectomy. Accessory thymus. Infective lesions.
U5	186 dys.	15 gm.	116 gm.	—	0.669 gm.	♀	—	0.036 gm.	0.178 gm.	Control. Suppurative foci in lungs.
U6	185 dys.	14 gm.	152 gm.	—	0.472 gm.	1.124 gm.	Active	0.030 gm.	0.202 gm.	Control. Normal organs.
U7	68 dys.	15 gm.	38 gm.	129 mm.	0.166 gm.	0.296 gm.	0	0.017 gm.	0.070 gm.	Control. Probably infected.

The bones and teeth were reserved for chemical examination and were not studied histologically. There were no gross changes.

The foregoing protocols show that rats may live without thymus tissue and in apparent health for at least 186 days. The differences in weight between the thymectomized rats and the controls are in all likelihood referable to infections, histological evidence of which was found in rats U1 and U4. Moreover, a sex difference of twenty-five grams at this age is to be regarded as normal. The great retardation of growth in rats U4 and U7 emphasizes the need of caution in attributing such effects to the removal of the thymus, rather than to incidental disease.

The following experiment offers further evidence in favor of the negative results of thymus extirpation.

Litter O.[2]—This litter consisted of six rats, upon four of which thymectomy was performed on July 17, 1913. During the following weeks all the animals received prophylactic injections of a vaccine of *B. enteritidis*. With the exception of one rat, O4, which developed bloody diarrhea and died after four weeks, all the rats of this litter gained steadily in weight and remained healthy. Rats O1, O2, and one of the controls, O5, were killed on Nov. 15, 1913, 120 days after the operation. Rat O3 and the remaining control, O6, were killed eleven days later. In none of the operated rats were there found rests of thymic tissue or accessory glands. It is unnecessary to give the detailed protocols, since a careful microscopic examination of the bones and teeth showed no abnormal conditions. The calcification of the dentine of the incisors is shown for comparison with that in rachitic rats in figures 1a and 1b.

The following protocols serve as examples of the second group, in which both operated and control animals showed distinct alterations in the bones and teeth.

Litter X.—This litter consisted of six rats, four of which were operated on Oct. 24, 1913, the remaining two being kept as controls. The estimated age at the time of operation was sixteen days.

Rat X1.—On Nov. 12, seventeen days after thymectomy, a portion amounting to about one fourth of the entire thymus of rat X6 was removed and implanted into the peritoneal cavity of X1. No ill effects followed the implantation until Dec. 1, when the rat was found lying upon its side, suffering from general convulsions. The forefeet were strongly flexed, the hind legs extended and paretic. The rat was killed with ether.

Autopsy.—Well nourished male; weight 36 gm. All operative wounds completely healed. Abdomen prominent, intestines distended. No peritonitis. Careful search shows no trace of implanted thymic tissue. Spleen small. No other visceral changes.

[2] Tabulated weights and measurements and weight chart of this litter are given in the previous article (1).

Cultures from heart blood remained sterile.

Microscopic Examination.—Liver normal, no necroses. Spleen follicles small. Many megakaryocytes. Kidney and pancreas normal. Testicular tubules lined with indifferent epithelium, showing no mitoses. No meningitis. Ganglion cells of motor area show no changes recognizable when stained with hematoxylin and eosin. Vesicles of thyroid large and filled with abundant colloid. Complete series of neck organs; every tenth section mounted. Later, intermediate sections including all suspicious areas were also examined. No undoubted masses of thymic tissue were found. A small nodule situated between the esophagus, descending aorta, and right bronchus, at first interpreted as a thymus rest, was subsequently concluded to be a lymph node. It showed a fairly distinct peripheral lymph sinus, and complete absence of large epithelial cell complexes and formed Hassall bodies. In and about the hypertrophic cervical lymph nodes were many eosinophilic polynuclear leucocytes. No mitoses were found in the parathyroids.

The bones and teeth were fixed in Orth's fluid and decalcified in 5 per cent. nitric acid.

No rachitic changes were present in the femur or tibia. The epiphyseal lines were narrow and regular; the layer of proliferating cartilage cells at the lower epiphysis of the femur were only two to three cells deep. The trabeculæ of the spongiosa were far apart and narrow, the osteoblasts flat and inconspicuous. The osteoid zone was extremely narrow, in many places not recognizable. The lymphoid marrow extends to the epiphyseal cartilage in the femur. The lower portion of the tibia is occupied by fatty marrow. In the lymphoid marrow of the femur, there are scattered areas of necrosis, similar to those found in infections with *B. enteritidis.*

Sections of the upper and lower incisors show nothing abnormal. The calcification of the dentine is complete, save for the normal narrow calcium-free zone.

The interesting point in this protocol is the onset of convulsions and a tetanoid condition after removal of the thymus and subsequent isotransplantation into the peritoneal cavity. It does not seem probable that the convulsions were due to intoxication following the absorption of the thymus tissue. At the time of death, the implanted fragment had apparently been entirely resorbed. Further, the experiment has been repeated in other rats, and in no case were similar symptoms observed. The presence of focal necroses in the bone marrow suggests an infection as a more probable cause for the nervous symptoms.

The absence of bone lesions, in spite of complete extirpation and lack of accessory tissue, is to be noted.

Rat X2.—On Nov. 12 a small fragment of thymus from rat X6 was implanted beneath the skin of the abdominal wall. The small nodule disappeared after a few days, and no remains of it were found at autopsy. There were no

convulsive symptoms or other evidence of illness following the implantation. The rat remained well, but was behind the remaining animals of the litter in size and weight. It was allowed to live 151 days after thymectomy, and was then killed together with control rat X5.

Autopsy.—Poorly nourished, eyegrounds pale, slight exophthalmos. Internal organs normal. In the thymic region there were five or six lymph nodes and fat; no gross remains of thymus.

The ribs show fairly marked deformity. The zone of growing cartilage is broad and translucent, and there is distinct nodular swelling at the junction of cartilage and bone. There are also several opaque, fusiform, whitish swellings along the shafts of the ribs. The femur is 5 mm. shorter than that of X5, the control, and thicker, but not noticeably deformed. The teeth show no gross changes, aside from a notching of the cutting surface of the upper incisors.

Microscopic Examination.—Neck organs cut in complete series. A small thymus IV is found at the lower pole of the right thyroid lobe. It contains no formed Hassall bodies, but there are many epithelial cells with large pale nuclei in the medullary portion. The smooth margin and the absence of follicles and peripheral lymph sinus easily distinguish the tissue from lymph gland. It can be traced through only six sections. There is a large accessory parathyroid on the left side, lying above and external to the thyroid. All parathyroids show numerous mitoses, one or two to each immersion field.

The liver is free from necroses. The splenic follicles are of moderate size and composed chiefly of large lymphocytes. The pulp shows groups of erythroblasts and fairly numerous eosinophilic mononuclears and megakaryocytes. There is evidently active hematopoiesis. In the lung there is hyperplasia of the peribronchial lymphatic tissue. Adrenal medulla shows intense chromaffin staining of cells and capillary contents. It does not differ from that of the control.

In the ribs the zone of proliferating cartilage cells is about 0.4 mm. in width (normal width about 0.1 mm.). By actual measurement with the projection apparatus, it is found to be about six times as broad as that of a normal control. It is composed of fourteen to sixteen swollen cells. The margin is uneven and irregular columns and groups of cartilage cells in which the ground substance stains like osteoid are found amongst the trabeculæ of the spongiosa. These are wide, irregular in their arrangement, and composed in the neighborhood of the epiphysis almost wholly of a homogeneous osteoid. Only the central portions take a bluish stain and show typical bone structure. The marrow spaces are narrow, and the marrow is fibrous. The osteoblasts are very high and conspicuous. The cortex is broad, and the marrow cavity of the shaft correspondingly reduced. The nodular swellings seen in the gross correspond to localized thickenings of the compact bone. The epiphyseal cartilage of the femur is very convoluted, and there are masses of swollen cells projecting amongst the trabeculæ of the spongiosa. The width of the proliferating zone varies. It is from six to twelve cells deep. The spongy bone is dense, the marrow spaces reduced in width.

The incisor teeth show pronounced changes in the calcification of the dentine, both in the upper and lower incisors. The essential feature is a defective and irregular calcification, with ingrowth of blood vessels into the calcium-free dentine. On the concave side of the upper incisor, there are alternating striæ of

calcified and calcium-free dentine. No lesions are found in the enamel epithelium. The changes in the teeth are illustrated in figures 2a, 2b, 2c, and 2d.

Rat X5.—Control, male. Killed with ether at the same time as X2.

Autopsy.—Well nourished, apparently healthy rat. No gross lesions of internal organs, ribs, femora, or teeth. Testes small, undescended.

Microscopic Examination.—Liver normal, no necroses. Splenic follicles about the same size as those of X2, and of similar structure. Mitoses are not very numerous. Megakaryocytes and eosinophils abundant in pulp. Adrenals show intense staining of chromaffin elements and of capillary contents. Testicular tubules for the most part devoid of differentiated spermatic cells, being lined with indifferent syncytium showing no mitotic figures. A few tubules show differentiation up to the formation of spermatids. The interstitial cells are hyperplastic. Thyroid vesicles slightly smaller, epithelium higher, than in the thyroid of X2. Parathyroids show mitoses (two or three in each section), but less numerous than in X2.

Two ribs were examined. One shows much more pronounced changes than the other. The lesions in the more severely affected rib are identical with those described in the rib of X2. The width of the growing cartilage is increased, and the disarrangement of the cartilage cells is extreme. There is an excessive amount of spongy bone in the region of the epiphysis, the trabeculæ having a wide osteoid margin. The marrow is fibrous in character. The femur also shows similar changes.

The incisors show a marked defect in the calcification of the dentine, with ingrowth of cells and blood vessels from the odontoblast layer, as shown in figures 3a, 3b, 3c, and 3d. The enamel epithelium of the upper incisors is interrupted at one point and folded in upon itself. The enamel in this region has fallen out.

The comparative weights and measurements of rats X2 and X5 are given in table II.

TABLE II.

Rat.	Duration of experiment.	Weight at operation.	Weight at death.	Length.	Spleen.		Adrenals.		Weight of testes.	Weight of thymus.	Remarks.
					Weight.	Length.	Absolute weight.	Relative weight.			
X2	151 dys.	19 gm.	86 gm.	150 mm.	0.333 gm.	27 mm.	0.022 gm.	$\frac{1}{3900}$ gm.	♀	—	Complete thymectomy. Small thymus IV.
X5	151 dys.	16 gm.	143 gm.	166 mm.	0.438 gm.	30 mm.	0.027 gm.	$\frac{1}{5300}$ gm.	0.374 gm.	0.143 gm.	Control. Cryptorchid.

There is a difference of weight of fifty-seven grams in favor of the control rat, and of sixteen millimeters in length. Both the operated rat and the control show marked lesions in the teeth and skeletal system.

The spontaneous occurrence of such lesions in the control rat

makes it highly improbable that they are in any way related to a loss of thymic function. Furthermore, if one is to ascribe the retardation in growth in rat X2 to the lack of thymus, one must assume that the presence of an accessory gland is not sufficient to ward off the effects. This would not coincide with Klose's assumption that such small lobules of accessory thymus tissue are capable of preventing the deprivation symptoms, and that the negative results of extirpation can be explained in this way.

The remaining rats of the litter, X3 and X4, and the control, X6, were killed 156 days after the operation.

Rat X3.—Large, well nourished male. Nothing abnormal noted in viscera, bones, or teeth. Many large lymph nodes in substernal region, but no gross remains of thymus.

Microscopic Examination.—Liver, spleen, adrenals, lungs, and kidneys normal. Testes show active spermatogenesis. Interstitial cells large, distinct, and numerous.

Neck organs cut in complete series. A lobule of thymus tissue is found lying along the trachea 1 cm. above the bifurcation. It is elongated and narrow, measuring approximately 1.5 by 0.3 by 0.075 mm. The thymus structure is distinct, although there are few fully formed Hassall bodies. At one pole are several vesicles lined with cuboidal epithelium, and containing basic staining colloid material. The right parathyroid lies external to the thyroid, and is irregular in shape, partially encircling the carotid. Mitoses are not infrequent.

In the upper incisors the calcification of the dentine is practically complete on the convex surface throughout the whole length. Near the base of the tooth, the border of the calcified portion is slightly wavy, and there are a few ingrowing blood vessels. The concave edge also shows good calcification of the dentine, although in the middle portion there are a few calcium-free streaks, and in places the calcium is deposited in globular form. The odontoblast layer is well formed through the entire extent of the pulp cavity on both sides. The enamel is wanting in the sections, but the emaloblasts are normal.

Normal chondrocostal junctions in several ribs examined. There are four to eight growing cartilage cells, regularly disposed. The osteoblasts are well formed. There is no excess of osteoid tissue.

Rat X4.—Thymectomy.

Autopsy.—Fairly well nourished female. No changes in internal viscera. No macroscopic remains of thymus. Definite lesions are seen in the ribs, similar to those described in X2. The chondrocostal junctions are swollen, and there is sharp angulation of the lower ribs. The zone of growing cartilage is broad, and the adjacent part of the shaft for a distance of about 2 mm. is greyish white and soft. Four of the ribs show on the pleural surface, about 1 cm. from the junction with the cartilage, pearly, cartilaginous swellings. Teeth and femora show no gross changes.

Microscopic Examination.—Liver, kidneys, and adrenals normal. Splenic follicles slightly smaller than those of the control, X6. Few mitoses. Many

eosinophils and much pigment in pulp. Lungs show many eosinophils in edematous tissue about larger blood vessels.

The upper incisors show changes similar to those described in rats X2 and X5, but somewhat less marked. The defective calcification of the dentine, on the convex surface, near the base of the tooth, is marked, about one third of the entire width being entirely calcium-free. In the middle portion, about two fifths of the dentine is uncalcified. Here, and also towards the tip, the calcium is deposited in laminæ. There is an abundant ingrowth of capillaries from the odontoblast layer. On the concave side, near the base, half the dentine is calcium-free. Towards the middle and distal portion, the calcification is still more incomplete, and in the form of alternating calcium-free and calcium-containing layers. The lower incisors show similar changes, especially marked on the concave surface.

The growing cartilage of the ribs presents an even convex surface towards the shaft. The rows of cartilage cells are six to ten cells deep. The spongiosa is composed of very broad trabeculæ with wide osteoid zones, and high osteoblasts. The pearly swellings observed in the gross correspond to localized thickenings of the compact bone.

The zone of growing cartilage of the femur at the lower epiphysis is convoluted and slightly increased in breadth, but not markedly irregular. The spongiosa is formed of coarse trabeculæ with a relatively broad osteoid zone and distinct osteoblasts.

Neck organs, cut in complete series, show no thymus tissue, with the exception of a minute accessory lobule below the inferior pole of the right parathyroid gland and lying within the capsule of the thyroid. This is composed of large cells with very pale nuclei, between which are scattered small cells of the lymphoid type. The lobule contains also a few eosinophils, and abundant brownish pigment. There are no Hassall bodies or large epithelial cell complexes. The tissue can be traced through only five sections. There are two parathyroids, both within the thyroid. The epithelial cells show fairly numerous mitoses, two or three to each section. The thyroid vesicles are small, save at the surface of the gland; the cells are columnar, but not infolded. The colloid is abundant, but vacuolated.

Rat X6.—Control.

Autopsy.—Large, well nourished rat, showing no changes in the internal organs. There is slight beading of the ribs, with a broad greyish zone adjacent to the cartilage. The femora and teeth appear normal.

Microscopic Examination.—Liver, kidneys, and adrenals normal. The splenic follicles are slightly larger than those of rats X3 and X4. Mitoses are not numerous. In the pulp are many erythroblasts and eosinophils. Some of the testicular tubules show active spermatogenesis with formed spermatozoa in the lumina. Most of the tubules, however, are lined with indifferent epithelium showing no mitoses. Interstitial cells are somewhat more abundant than in the testis of rat X4.

In the upper incisors the odontoblasts are well formed, extending through the entire length of the pulp. Calcification of the dentine is not abnormal, save that the odontoblast layer is rather wavy. The uncalcified predentine is narrow.

Ribs and femur show nothing abnormal.

The comparative weights and measurements of rats X3, X4, and X6 are summarized in table III.

TABLE III.

Rat.	Duration of experiment.	Weight at operation	Weight at death.	Length.	Spleen.		Adrenals.		Weight of testes.	Remarks.
					Weight.	Length.	Absolute weight.	Relative weight.		
X3	156 dys.	19 gm.	195 gm.	199 mm.	0.447 gm.	31 mm.	0.027 gm.	$\frac{1}{7240}$	1.382 gm.	Incomplete thymectomy.
X4	156 dys.	16 gm.	110 gm.	165 mm.	0.311 gm.	31 mm.	0.028 gm.	$\frac{1}{3947}$	♀	Complete thymectomy. Minute thymus IV.
X6	156 dys.	20 gm.	180 gm.	205 mm.	0.542 gm.	34 mm.	0.021 gm.	$\frac{1}{8571}$	0.440 gm.	Control. Hypoplasia of testicles.

Rat X4 offers a further illustration of the fact that rats may survive complete extirpation for many months without becoming cachectic. It seems improbable that the minute accessory gland, less than fifty microns in diameter, could take over the functions of the entire thymus without showing evidence of active hyperplasia.

The rachitic lesions in this rat were marked, and as will be evident from subsequent protocols, this disease in rats is frequently, though not always, accompanied by a retardation of growth. There is, therefore, little ground for attributing the diminution in size and weight, as compared with the control, to the lack of thymus. The normal sex difference must also be taken into account at this age.

Litter Y.—This litter, consisting originally of eleven rats, was born in the laboratory of stock animals, all the rats previously studied having been obtained from Granby, Mass. The rats were undersized, and stood the operation poorly, only three thymectomized and three controls surviving.

Rat Y2.—Thymectomized Oct. 27, 1913. Weight 10 gm. Gained slowly and remained undersized. Killed on Mar. 13, 1914, 137 days after extirpation of the thymus.

Autopsy.—Internal organs normal save for suppurative foci in the lower lobe of the right lung. Testes large and descended. No alterations of the bones and teeth were recorded.

Microscopic Examination.—Lungs show bronchiectases and bronchopneumonia. Active spermatogenesis. Interstitial cells not numerous. Liver, adrenals, and kidneys normal. Spleen shows active blood formation.

Extirpation of thymus probably complete. The only suspicious tissue lies in a lobule of fat near the hilus of the left lung, where there is a circumscribed collection of mononuclear cells with a few larger elements, which can be traced

through sixteen sections. There are no epithelial cell complexes or Hassall bodies, but the tissue does not have a peripheral lymph sinus or follicles. It is probably a newly forming lymph node. The thyroid is composed of large vesicles with abundant colloid and flat epithelium. In the fat tissue external to it, are a few closed ducts lined with high cylindrical epithelium (postbranchial ducts).

The line of growing cartilage of the ribs is broad, with a convex, irregular surface directed towards the shaft. It is composed of eighteen to twenty cells in rows, with masses of cartilage cells projecting amongst the trabeculæ of the spongiosa. These are numerous, broad, with a wide osteoid margin, and high conspicuous osteoblasts. The marrow spaces in the region of the cartilage are narrow, the vessels hyperemic and surrounded by spindle cells.

Upper epiphysis of tibia. No definite rachitic changes. The zone of growing cartilage is slightly narrower than that of the control. The cells are regular in their arrangement, as are the trabeculæ. Bone formation is very active. The osteoid zone about the trabeculæ is slightly increased in width. This is especially clear in the epiphyseal center of ossification.

The epiphyseal line of the femur is regular on the whole, but there are a few projections of cartilage amongst the trabeculæ of the spongiosa. The osteoid is increased in amount.

The calcification of the dentine of both upper and lower incisors is somewhat defective, but there is no vascularization of the calcium-free dentine. The enamel epithelium is not altered.

Much more striking were the skeletal and dental changes in rat Y8, the protocol of which follows.

Rat Y8.—Thymectomy on Nov. 1, 1913. Weight at operation 14.6 gm. Following the operation, and until the rat was killed on Mar. 2, 126 days after extirpation of the thymus, it lagged far behind the remaining rats of the litter in weight and size. The hair was rough and sparse; the head seemed relatively large as compared with the body. On Jan. 17, it was noted that the upper incisors were very long and thick as compared with the lower, which were small and delicate. On Feb. 28 the right upper incisor was broken off just beyond its emergence from the gum.

Autopsy.—Poorly nourished, stunted male. Weight 42 gm. The internal viscera were normal, with the exception of the right lung, which was atelectatic in its upper portion. No gross remains of thymus. The thorax is greatly deformed by the presence of a deep groove along the line of the chondrocostal junctions. From the pleural surface there are seen, especially on the lower ribs, nodular protuberances at corresponding points. There is thus a pronounced rachitic rosary. The bones of the lower extremities are softer and more pliable than normal, and there is distinct swelling about the epiphyses of the knee-joints.

Cultures from the heart blood and spleen on slant agar remained sterile.

Microscopical Examination.—Liver free from necroses. Splenic follicles large and sharply circumscribed. Many of the lymphoid cells are of the large mononuclear type. Mitoses are scarce, and there are no distinct germinal centers. In the pulp there are many islands of normoblasts and groups of myelocytic eosinophils. Adrenals show the usual intense chromaffin staining. Testicular tubules are lined with four or five rows of cells, most of them spermatoblasts

in active division. There are a few spermatids, but no ripe spermatozoa. The appearance is that of a testis just before the onset of spermatogenesis. Some of the bronchi contain dense collections of eosinophils. The pulmonary arteries are thickened, their lumina contracted, the muscular coat appears hypertrophied. Some of the vessels are practically obliterated.

Complete series of neck organs. There is no tissue which can be identified as thymus. There are no accessory thymus lobules. The parathyroids are large; no mitoses are found. Thyroid shows usual structure with abundant colloid.

The upper incisors were sectioned through the fractured stump. The pulp is hyperemic and largely necrotic. The calcification of the dentine is both irregular and incomplete. The line of junction between odontoblasts and predentine is sinuous, and in many places the odontoblast cells penetrate the uncalcified tissue. The calcified portion of the dentine is laminated, the calcium being in the form of coarse globules. In another section there are alternating zones of calcified and calcium-free dentine. Isolated cells and occasionally capillary loops reach the outer, imperfectly calcified zone. Sections through the unfractured upper incisor show also defective calcification. Near the root, there is the same stratification as in the fractured tooth. In the middle portion, just after it emerges from the socket, the section passes outside the pulp canal. In this region there is a large area of calcium-free dentine. Near the convex surface, within the calcified strata, there are a number of globular yellowish masses. In sections stained with thionin-picric acid, it is seen that the dentine filaments are interrupted at the site of these globular deposits. Near the root, the congested pulp vessels penetrate the uncalcified dentine as papillary ingrowths. About one half of the dentine on the convex surface of the lower incisors is free from calcium. The odontoblasts are poorly formed and irregular. There is no marked ingrowth of blood vessels. On the concave side the calcification is still more imperfect.

The zone of growing cartilage of the humerus at the lower epiphysis is widened, and irregularly prolonged amongst the trabeculæ. These are very broad and the marrow spaces correspondingly narrow. The trabeculæ are composed in the neighborhood of the epiphysis of a narrow, ossified central portion, distinctly laminated, with angular bone corpuscles, surrounded by a very broad, homogeneous, osteoid zone in which the nuclei of the bone corpuscles are larger and more rounded, and quite irregularly distributed. The osteoblasts are conspicuous and well formed. There is no increased osteoclastic resorption. The changes at the lower epiphysis of the femur are similar in character, but more pronounced than in the humerus. Numerous sections from four different ribs show similar changes. There is marked deformity at the junction of cartilage and bone, where the rib measures 3 mm. in diameter. The junction of the cartilage with the shaft is in most places sharply marked off. There is a large central mass of hypertrophic cartilage, in some places twenty-five cells deep, and two shorter lateral spurs. Near the pleural surface, smaller irregular groups of cartilage cells are surrounded by osteoid-staining matrix. The adjacent cancellous bone is composed of stout trabeculæ formed entirely of pink staining osteoid, separated by very narrow clefts and spaces. They are ranged, in the region where the rib is sharply angulated, at right angles to the cartilage columns.

The distribution of the bone corpuscles through this mass of osteoid is haphazard. Farther along the shaft, the trabeculæ of the spongy bone follow the long axis of the rib. They are all greatly thickened, as is the corticalis, and well calcified only in their central portions. In thionin-picric acid-phosphotungstic acid-stained preparations, the broad osteoid, blue staining zone appears with great distinctness. The marrow cavity is much reduced in width. Near the epiphysis the marrow is fibrous; further along, it has the normal lymphoid appearance.

Two control rats of the litter, Y7 and Y9, showed alterations in the teeth and bones comparable with those observed in the thymectomized rats, though somewhat less pronounced. A third control, Y10, which had complete visceral transposition, was not histologically examined.

Rat Y7.—Control. Killed Apr. 7, 1914. Poorly nourished female, weighing 115 gm. Internal organs show no lesions, with the exception of the lung. The right middle lobe is converted into a mass of cheesy abscesses, separated by scar tissue. The ribs show slight beading at the chondrocostal junctions, with angular deformity in the region of the diaphragmatic attachment. The zone of growing cartilage is broad. No gross changes in femora or teeth.

Microscopic Examination.—Internal viscera, aside from lung lesions, show nothing abnormal.

In the upper incisors calcification of the dentine is imperfect, both on the convex and on the concave surfaces. The odontoblast layer is broad, but ill defined, and on the concave side cells are seen to penetrate the calcium-free dentine for varying distances. Both surfaces of the lower incisors show alternating layers of calcium-containing and calcium-free dentine. The calcium is deposited in the form of coarse globules.

TABLE IV.

Rat.	Duration of experiment.	Weight at operation.	Weight at death.	Length.	Spleen.		Adrenals.		Weight of testes.	Remarks.
					Length.	Weight.	Absolute weight.	Relative weight.		
Y2	150 dys.	10 gm.	85 gm.	145 mm.	26 mm.	0.220 gm.	0.013 gm.	$\frac{1}{5538}$	1.483 gm.	Complete thymectomy.
Y8	121 dys.	14.5 gm.	42 gm.	127 mm.	25 mm.	0.312 gm.	0.020 gm.	$\frac{1}{2100}$	0.332 gm.	Complete thymectomy.
Y9	150 dys.	16 gm.	118 gm.	170 mm.	32 mm.	0.380 gm.	0.030 gm.	$\frac{1}{3933}$	1.980 gm.	Control. Abscesses of lungs.
Y7	168 dys.	16 gm.	115 gm.	157 mm.	34 mm.	0.333 gm.	0.043 gm.	$\frac{1}{2674}$	♀	Control.

The growing cartilage of the femur is narrow, being composed of only two to four rows of cells in the lower epiphysis. Both in the compact and in the spongy bone, the osteoid margin is very broad and distinct, especially about the

perforating vessels. The ribs show no deformity in the sections examined, but there is a distinct increase in the width of the osteoid zone about the trabeculæ.

Rat Y9.—Control, male. Killed Mar. 13, 1914. Weight 118 gm. There were no lesions of the internal viscera. The alterations of the teeth were insignificant (slight lamination of the calcium on the convex surface of the lower incisors), but there was distinct irregularity and thickening of the cartilage at the lower epiphysis of the femur and at the upper epiphysis of the tibia. The single rib examined was practically normal.

The comparative weights and measurements of litter Y are given in table IV.

To the foregoing protocols of thymectomized and control rats may be added the brief description of two unoperated rats taken from the stock cages, which showed lesions in the bones and teeth identical with those described above.

The first of these (rat 50) was greatly stunted; there was a marked disproportion between the size of the head and that of the body. The approximate weight was 50 gm. The hair was rough and sparse. The teeth were somewhat chalky, but not greatly altered. There was an extreme deformity of the thorax (figure 5), resembling that noted in rat Y2. There was also marked kyphosis and lateral curvature. There were many whitish nodules along the shafts of the ribs, especially in the paravertebral lines. The ribs were very soft, and on cross-section the marrow cavity seemed almost obliterated owing to the great thickening of the cortex of the bone. At the chondrocostal junctions were nodular projections several millimeters in thickness. The femora were short, and very broad in the region of the epiphyses. The upper third of the tibiæ was bowed anteriorly.

Microscopic Examination.—The superficial layer of dentine of the upper incisors is well calcified; in the middle zone, the calcium is in the form of globular masses of large size, and the inner zone, comprising about one third of the total thickness of the dentine, is calcium-free. Towards the apex the calcification is more complete. The lower incisors exhibit more striking changes, especially on the concave surface where about half the dentine is free from calcium. The vessels of the pulp are extremely congested, and the odontoblast layer is broken up in many places by fresh capillary hemorrhages. Towards the apex of the tooth, the layer of odontoblasts becomes entirely disorganized, with the formation of numerous ragged cavities, possibly the effect of gas formed during decalcification. The enamel epithelium is intact save in one place, where the cells are destroyed by hemorrhage.

The zone of proliferating cartilage of the ribs is composed of irregular masses of cells which fail to show the normal alignment into columns, and which extend a variable distance into the adjacent trabeculæ. The neighboring part of the rib is composed of a knob of anastomosing columns separated by very narrow Haversian spaces. The distinction between osteoid and calcified bone is not sharply brought out in the sections, but the rounded form of the bone corpuscles and their highly irregular distribution suggest deficient calcification. The osteoblasts form a continuous flat row covering the trabeculæ. There is no increased

lacunar resorption. Beyond this knob of tissue the bone is sharply bent upon itself. A tongue of cartilage grows into this angle from the external surface of the periosteum. The cortex of the rib is greatly thickened and formed of parallel trabeculæ which encroach upon the medullary cavity, which is correspondingly narrowed. The marrow is normal, save in the region of the junction where it is fibrous (figure 6).

In order to determine the possible infectious character of this disease, four half grown and apparently healthy rats were inoculated with a suspension of bone marrow from rat Y8. One of the inoculated rats, killed twenty-eight days after subcutaneous injection, showed striking alterations in the bones and teeth. There was in this rat no gross deformity of the thorax, but an evident widening of the translucent zone of proliferating cartilage, and the adjacent bony part of the ribs was opaque and slightly thickened. The femora were broad and thick, and so soft that they could easily be cut with scissors. The tibiæ were bowed anteriorly.

Changes in the teeth were first noted four days before the animal was killed. The basal portions of the upper incisors were found to be distinctly swollen, translucent, greyish, and rounded, sharply marked off from the distal third which was opaque and chalky white. During the next few days, the swelling and translucency extended almost to the tip of the tooth. The lower incisors were also less opaque than normally.

Microscopic Examination.—No changes were found in any of the internal viscera. The marked defect and irregularity of the dentine is shown in figures 4a, 4b, 4c, and 4d. It is seen that on the convex surface of the upper incisors the irregularity in the arrangement of the odontoblasts is such that these have apparently given rise to predentine both on their external and internal surfaces. They are thus disposed in a row on both sides of which is uncalcified dentine, and are connected by numerous cell strands with the pulp. The concave side of the tooth is calcium-free throughout its entire extent, and is pierced in many places by isolated spindle cells and occasionally by capillaries. The changes in the ribs and femora consisted merely of an increase in the width of the osteoid margins. No definite alterations were found in the cartilages.

DISCUSSION.

The foregoing protocols show that lesions of the bones and teeth were observed in thymectomized rats, in controls of the same litters, in stock animals, and in a rat inoculated with bone marrow from a thymectomized rat with lesions of the skeletal system.

There is little question that these lesions resemble closely those described by Morpurgo (5), in 1900, as infective osteomalacia of white rats. In his subsequent investigations, extending over many years, Morpurgo showed that in young animals, the spontaneous disease took the form of rachitis.

From the spinal cord of a spontaneously diseased osteomalacic rat, and later, from the spleen, liver, kidneys, and bones, Morpurgo isolated a Gram-positive diplococcus with a tendency to form chains in fluid cultures. With pure cultures of this microörganism, Morpurgo was able to produce rachitic lesions in young rats, and osteomalacia in adult rats in which epiphyseal growth had been completed.

The clinical course of the disease varied in different rats, in part owing to variations in the virulence of the cultures, in part apparently to variations in the susceptibility of individual animals. In some cases the rats became greatly emaciated, and died from the disease. Other animals lived for over a year, in which case the bone lesions showed evidence of healing.

The deformities produced also varied widely in different rats. They were found especially in the vertebral column, the thorax, the scapulæ, the posterior extremities, and the pelvis,—more rarely in the anterior extremities and the maxillary bones. The vertebral column was lordotic and scoliotic in the dorsal region, kyphotic in the sacral and cervical portions. The thorax was much depressed along the insertion of the pectoral muscles, and along the line of depression the ribs were enlarged in consequence of callus formation following infractions or complete fractures. Small beads of callus were also seen along the paravertebral line, or scattered irregularly along the course of the ribs. The cartilages were also tortuous, nodose, and yellowish.

The femora were rarely curved or bent, and only when the presence of callus indicated a previous fracture. The diaphysis was often greatly enlarged, the medullary cavity eccentric or narrowed. The tibia was often bent at an angle between the upper and middle thirds.

The microscopic examination of the bones disclosed a picture which was identical with that seen in human rickets or osteomalacia. Schmorl and others have accepted the morphological identity of the lesions in the rat with those of human rachitis.

I have not found in Morpurgo's numerous papers any reference to changes in the teeth. Weichselbaum (6), however, reported finding lesions in the teeth in three spontaneously rachitic rats, which he describes as follows:

"The dentine of the incisors nowhere shows a uniformly calcified zone, but the zone adjacent to the odontoblasts, *i. e.*, the younger layer, was completely bereft of calcium, and thus appeared pale red in hematoxylin-eosin preparations, whereas the older layers were either wholly calcium-free or unevenly calcified, in which case they contained numerous blue dentine globules separated by pale, so called interglobular spaces. One sees further that from the odontoblast layer, capillary loops penetrate more or less deeply into the dentine. In one of the two rats one of the upper teeth was broken off."

Weichselbaum calls attention to the close resemblance of these changes found in the spontaneous rachitis of rats, to the lesions found by Fleischmann (7) in the teeth of human rachitic subjects, and especially to the dental changes described by Erdheim (8) and Toyofuku (9) as following parathyroidectomy in rats.[3] Hohlbaum (10), also, confirming Erdheim's findings, noted in two spontaneously affected rachitic rats identical lesions in the teeth. Furthermore, Iselin (11), in his study of the effects of parathyroid extirpation, found rachitic-like changes in the epiphyses of those rats which survived for a longer period, and attributed them to the loss of parathyroid function.

Defective calcification of the dentine, and penetration of the uncalcified tissue by blood vessels and isolated cells from the odontoblast layer, regarded by Erdheim and his successors as characteristic of parathyroid deprivation, were observed also in many of the rats described above. In one rat, there occurred a spontaneous fracture of the tooth. Less marked in my rats was the infolding and disarrangement of the emaloblast layer; but this appears to be of secondary importance also in the lesions attributed to parathyroidectomy, and when present is probably due to mechanical causes.

The question arises, therefore, whether Erdheim, who was familiar with the spontaneous rachitic lesions of rats, was justified in attributing these changes to parathyroid insufficiency. Erdheim (12) has brought forward his transplantation experiments as crucial proof of the part of the parathyroids in normal calcification. The *"Transplantations-streife"* is believed to correspond exactly in its localization with the period of functional activity of the implanted parathyroid tissue.

This would be entirely convincing were it not for the fact that such alternating layers of calcium-containing and calcium-free den-

[3] This resemblance was accepted by Erdheim, who studied Weichselbaum's specimens.

tine not infrequently occur as spontaneous rachitic lesions. They are associated, no doubt, with remissions and exacerbations of the disease, such as have been described by Morpurgo. Figure 3 illustrates the striate deposition of the calcium in the incisor of a spontaneously rachitic rat.[4]

Without wishing to discredit the work of Erdheim, supported as it is by other facts indicating an influence of the parathyroids upon calcium metabolism, one cannot but emphasize the identity of these lesions attributed to the loss of parathyroid function, with those occurring in the spontaneous rickets of rats.

As regards the infectivity of the disease and the nature of the inciting organism, studies made in coöperation with Dr. J. G. Hopkins have not as yet led to conclusive results. The occurrence of rachitic lesions in a rat, examined twenty-eight days after inoculation of bone marrow from a spontaneously rachitic animal, is of doubtful significance, since many of the stock animals killed at this time were found to be rachitic. The recent experiments of Koch (13), in which typical rachitic lesions were produced in puppies by the intravenous injection of streptococci, indicate that the infective factor has to be reckoned with in experiments dealing with the effect of extirpation of ductless glands upon bone development in dogs as well as rats.

Sufficient evidence, we believe, has been brought forward to show that the bone changes described by Klose are in no way related to the loss of the thymus gland, but occur as the result of spontaneous disease. It is difficult indeed to understand why Klose should believe that his "experiments on rats acquire fundamental significance in view of the experiments of Morpurgo";[5] and why, in the same article, he should imply that rats are amongst those species which are practically immune to spontaneous rickets.[6]

As regards alterations in the weights and microscopical structure of the adrenals, testes, and spleen, which might point to a correlation between the thymus and these organs, nothing has been found to

[4] This appearance is pictured in figure 4, plate 6/7, of Toyofuku's paper (9), where it occurred in a rat, forty-six days after parathyroidectomy, in which no transplantation had been performed.

[5] Klose, *loc. cit.*, p. 4.

[6] Klose, *loc. cit.*, p. 7.

modify the negative conclusions reached in the previous paper. An analysis of the additional figures given therefore seems unnecessary.

CONCLUSIONS.

Rachitic changes in the bones and teeth occur in young albino rats as the result of spontaneous disease, possibly of infective origin. This disease is often, but not always, accompanied by an arrest of growth and malnutrition.

There is no valid reason for attributing such lesions to the loss of thymic function. In some of the rats showing the disease, an unusual number of mitoses were found in the parathyroid glands, but no evidence of injury to these structures.

It has not been found possible to confirm the statements of Klose and Magnini as to the fatal effects of complete thymus extirpation in rats.

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EXPLANATION OF PLATES.

Plate 22.

Fig. 1a. Rat O1. Thymectomy. Killed after 120 days. Upper incisor, convex side. D1 = calcium-free dentine. D2 = calcified dentine. O = odontoblasts.

Fig. 1b. Same tooth, concave side. Normal relations between calcified and uncalcified dentine.

Fig. 2a. Rat X2. Thymectomy; accessory thymus. Killed after 151 days. Upper incisor, convex side, about middle of tooth. Defective calcification of dentine, ingrowth of blood vessels from odontoblast layer. D3 = incompletely calcified dentine. C = capillary.

Fig. 2b. Concave side of same tooth. Same designations.

Fig. 2c. Lower incisor, concave side.

Fig. 2d. Lower incisor, convex side.

Plate 23.

Fig. 3a. Rat X5, control. Killed at same time as X2. Upper incisor, convex side.

Fig. 3b. Upper incisor, concave side. Deposition of calcium in layers.

Fig. 3c. Lower incisor, convex side.

Fig. 3d. Lower incisor, concave side. Alternating striæ of calcium-containing and calcium-free dentine.

Fig. 4a. Rat Y8(3). Injected subcutaneously with suspension of bone marrow of rachitic rat Y8. Killed after 28 days. Upper incisor. Incomplete calcification of dentine. Irregular disposition of odontoblasts. I = islands of uncalcified dentine. P = pulp.

Fig. 4b. Upper incisor, concave side. Dentine wholly calcium-free. Odontoblast layer indistinct.

FIG. 4c. Lower incisor, convex side.

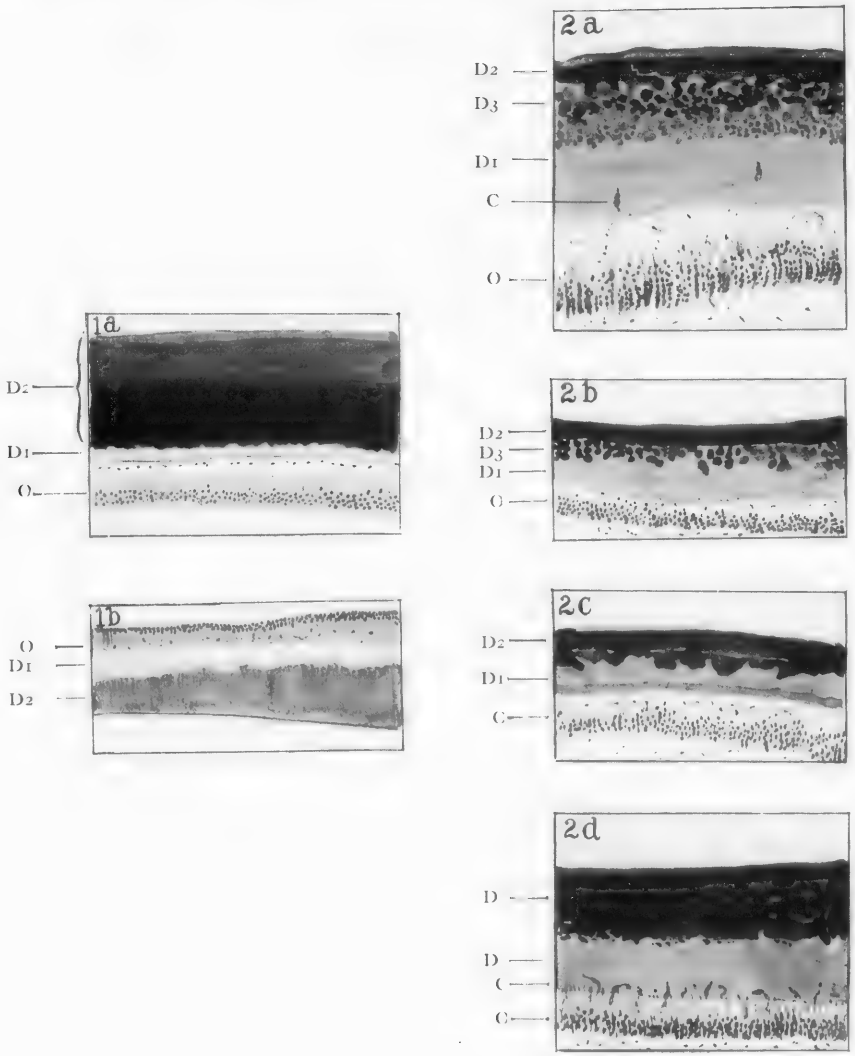
FIG. 4d. Lower incisor, concave side. Dentine entirely uncalcified and convoluted. Ingrowth of odontoblasts and capillaries.

PLATE 24.

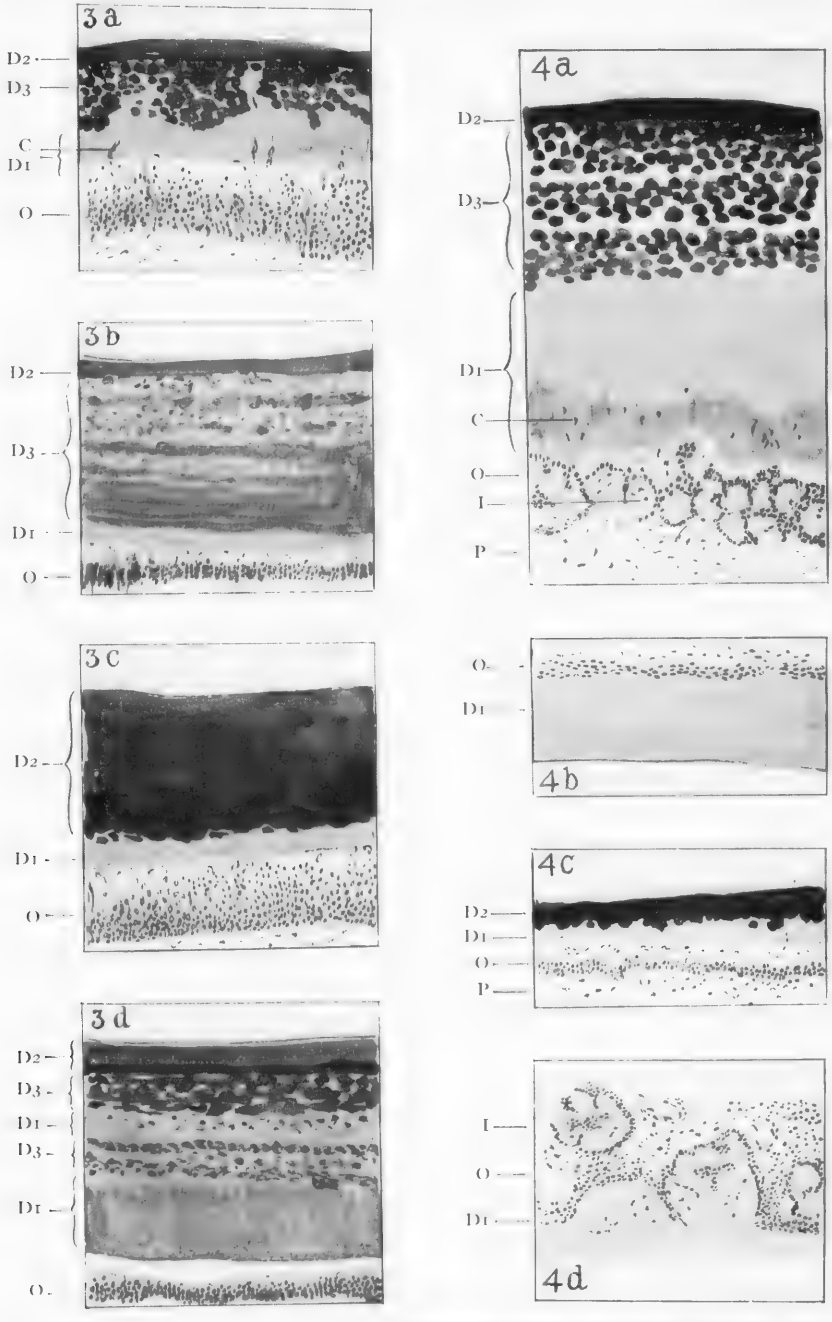
FIG. 5. Rat 50. Spontaneous rachitis. View of right half of thorax and vertebral column.

PLATE 25.

FIG. 6. Rat 50. Section of rib, low power drawing. K = zone of proliferating cartilage. K1 and K2 = islands of cartilage. O1 = osteoid tissue. O2 = calcified bone. P = ingrowth of periosteum. V = thickening of periosteum (old callus?). M = medullary cavity.



(Pappenheimer: Effects of Extirpation of Thymus in Rats.)



(Pappenheimer: Effects of Extirpation of Thymus in Rats.)

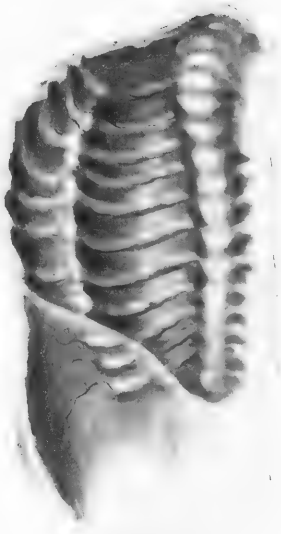


FIG. 5.

(Pappenheimer: Effects of Extirpation of Thymus in Rats.)

FIG. 6. (Pappenheimer: Effects of Extirpation of Thymus in Rats.)

THE CALCIUM CONTENT IN BONES AND TEETH FROM NORMAL AND THYMECTOMIZED ALBINO RATS.*

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This paper deals with the proportions of calcium in the bones and teeth from rats used by Dr. Pappenheimer in the experiments described in the preceding paper.[1] After the conclusion of the autopsy by Dr. Pappenheimer, and the removal from the rat of any parts required by him, we took all the teeth and also the femurs and tibias from each animal. The teeth individually were carefully pulled out of place after the grip of the bones and membranes that held them had been broken by suitable paring and excision. Each tooth was cleaned with filter paper. Adherent muscle was cut from the leg bones, which were then gently scraped clean with a scalpel.

After free exposure to the air, for prompt desiccation at room temperature, the teeth and bones were separately crushed and pulverized in a deep porcelain mortar. When, for any reason, we were unable to obtain all the teeth with both the femurs and the tibias from an animal, we used these materials from one side only, and proceeded on a 50 per cent. basis, in this way preventing any analytic irregularities that might have resulted from the use of masses that were not anatomically equivalent in kind and proportion.

After the pulverized material had been uniformly mixed and dried to constant weight at 100° to 110° C., an accurately weighed portion of it, practically all the material in each case, was decomposed in a large Kjeldahl digestion flask of Jena glass, with a mixture of equal parts of concentrated sulphuric and nitric acids (Neumann's method).[2] The clear, colorless, concentrated, acid solution, freed from most of the mechanical excess of residual sulphuric acid, was then diluted to 500 cubic centimeters or one liter, and kept in tightly stoppered bottles. Calcium was determined in duplicate by

* Received for publication, August 10, 1914.

[1] Pappenheimer, A. M., *Jour. Exper. Med.*, 1914, xx, 477.

[2] Thierfelder, H., in Hoppe-Seyler, F., Handbuch der chemischen Analyse, 8th edition, Berlin, 1909, 539.

McCrudden's method[3] in 100 cubic centimeter portions of each solution prepared in this way.

We are indebted to Dr. Pappenheimer for the accompanying tabular statement of his data (table I), in which he has inserted our percentage results for calcium in the water-free bones and teeth.[4]

TABLE I.

Data Pertaining to Albino Rats Described in the Preceding Paper.[5]

Rat.	Thymectomy.	Duration of experiment, in dys.	Weight at death, in gm.	Rachitic lesions.		Percentage of calcium.[6]		Remarks.
				Bones.	Teeth.	Bones.	Teeth.	
U_1	Complete	185	97.3	None	None	22.61	27.48	No histological examination of bones or teeth.
U_3	Complete	186	111.0	None	None	24.01	28.97	No histological examination of bones or teeth.
U_5	Control	186	116.0	None	None	23.16	27.93	No histological examination of bones or teeth.
U_8	Control	185	152.0	None	None	22.74	28.29	
X_3	Incomplete	156	195.5	None	Slight	21.37	26.51	
X_4	Complete	156	110.5	Moderate	Slight	20.89	28.61	Minute thymus IV.
X_5	Control	151	143.0	Marked	Marked	18.92	—	
X_6	Control	156	180.0	None	Slight	22.84	28.12	
Y_2	Complete	150	85.0	Marked	Moderate	17.85	28.53	
Y_7	Control	168	115.0	Moderate	Moderate	23.14	27.09	
Y_9	Control	150	118.5	Moderate	Moderate	18.68	27.23	
W_2	Complete (?)	192	240.0	None	None	24.40	28.31	Series of neck organs incomplete.
W_4	Control	192	240.0	None	None	23.75	28.99	
Z_1	Incomplete	166	145.0	None	Very slight	22.29	28.43	
Z_4	Control	166	123.0	None	None	23.13	27.84	
50	Stock	—	—	Marked	Slight	23.41	26.44	Inoculated with bone marrow from spontaneously rachitic rat.
53	Stock	—	90.0	Moderate	Marked	18.15	—	Spontaneous rachitis.
55	Stock	—	75.0	Moderate	Marked	20.66	25.49	Inoculated with bone marrow from spontaneously rachitic rat.

[3] McCrudden, F. H., *Jour. Biol. Chem.*, 1911, x, 187. Neuberg, C., Der Harn, sowie die übrigen Ausscheidungen und Körperflüssigkeiten, Berlin, 1911, i, 168.

[4] We did not remove the lipins, on the assumption that variations in their proportions could not be great enough materially to affect the data obtained on a water-free basis.

[5] Pappenheimer, A. M., *loc. cit.*

[6] Calcium content is expressed as calcium (Ca), not as lime (CaO).

Data pertaining to average percentage amounts of calcium in the water-free bones and teeth, classified for various groups of the rats, are given in table II.

TABLE II.

Data Pertaining to the Content of Calcium, in the Bones and Teeth, for Various Groups of the Rats Referred to in Table I.

Groups of rats.	Bones.	Teeth.
	Percentage of calcium.	
1. Non-rachitic and slightly rachitic	23.03	27.99
2. Moderately rachitic and markedly rachitic	20.21	27.08
3. Completely thymectomized (excluding W_2)[7]	21.34	28.40
4. Control and incompletely thymectomized[7]	22.00	27.83
5. Rachitic (stock)	20.74	25.96

The foregoing chemical data agree, in the main, with the corresponding histological findings reported in Dr. Pappenheimer's paper. Our results indicate that the bones and teeth of the rats which were found by Dr. Pappenheimer to be moderately rachitic and markedly rachitic, contained in general less calcium than those from the non-rachitic and slightly rachitic animals. The lack of any particular trend of calcium content in the bones and teeth from the thymectomized rats, as shown by both the individual results in table I and the group averages in table II, makes it impossible to ascribe to thymectomy, in these animals, any special influence upon the processes of calcification in the bones and teeth subjected to analysis. The validity of this negative conclusion is emphasized by the low values for calcium content in the bones and teeth from the unoperated rachitic (stock) animals. That such spontaneously rachitic rats happened to be among the number selected for Dr. Pappenheimer's experiments, and that rachitic lesions observed in the thymectomized animals were due to causes independent of thymectomy, are necessary inferences under the circumstances. It is possible, of course, that determinations of phosphate content might have afforded indications of a special influence of thymectomy.

[7] Rat W_2 (table I) evidently belongs to group 4 in this table. If the figures for the calcium content pertaining to rat W_2 are added to those of group 4 in this table, the average values for calcium become 22.22 and 27.87 per cent., respectively.

It is assumed by Dr. Pappenheimer and by us that the mixed food of the rats, which was given in abundance and eaten freely, was ample in quantity, in quality, and in metabolic availability for the maintenance of the processes of normal calcification in each animal. One cannot be certain of such an outcome in experiments of this kind, however, unless the animals are individually maintained under suitably controlled metabolic conditions on diets of definite composition, of known quantity, and of proved nutritional adequacy. We require also, for correct interpretations of analytic data on calcification, more exact chemical knowledge of the variations in the composition of the bones and teeth of normal albino rats of different ages under given metabolic conditions. It is our intention to investigate in this laboratory, as a part of a plan of research in progress, the facts in these particular connections, with special reference to the possible establishment of definite physiological standards on which further experimental work with albino rats may be conducted more accurately.

The analyses reported in this paper were conducted during the progress of experiments by us on the influence of thyroparathyroidectomy on dentition in albino rats, under the auspices of the Dental Society of the State of New York. In the execution of the analyses pertaining to that work, and also of those reported here, we have enjoyed the assistance of our associates, Dr. Alfred P. Lothrop and Mr. William A. Perlzweig.

THE INFLUENCE OF VARIOUS SUBSTANCES ON THE GROWTH OF MOUSE CARCINOMA.*[1]

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We carried out the following experiments in order to test the action of various classes of substances on the growth of mouse carcinoma. We used in our experiments a mouse carcinoma, transplanted by us through many generations from a spontaneous tumor sent from Granby, Mass., about seven or eight years ago. In this tumor the cells after a certain initial period of growth are arranged in solid alveoli. Later caseous necrosis sets in in the central parts of the tumor. The tumor was inoculated into American mice weighing approximately sixteen grams. The inoculation was positive in 80 to 90 per cent. (on the average in 82 to 83 per cent.) of the animals. In testing the effect of a certain substance on tumor growth, the mice were injected on the 9th, 10th, 11th, and 12th days after inoculation. The large majority of all injections were done intravenously, the veins of the tail being used. In some cases the effect of subcutaneous injections was compared with the intravenous administration. Two methods were used in determining

* Received for publication, July 16, 1914.

[1] The greater part of this work was carried out during the year 1912 and during the first half of 1913. Since then we enlarged the scope of our work by making additional experiments of a quantitative character. Previous references to these experiments are found in the following communications: Loeb, L., and Fleisher, M. S., *Interstate Med. Jour.*, 1912, xix, 476; 1913, xx, 569; *Jour. Am. Med. Assn.*, 1913, lx, 1857.

In some of our previous papers references have been made to the literature on this subject as far as it preceded our own work. Since we have published our first report a number of communications by various authors have appeared which dealt with the effect of isolated substances rather than with a comparative study of various groups of substances which we undertook. A review of the literature on this subject is given by Keysser, F., *Ztschr. f. Chemotherap., Ref.*, 1914, ii, 1325.

the effect of the injections. (1) On the ninth day after inoculation a drawing of each tumor was made; on the twelfth and thirteenth days another drawing was made. This permitted us to determine with a sufficient degree of certainty whether a tumor had grown very much during this period or whether it had been retarded in its growth. This method was sufficient for determining whether or not a certain substance exerted a decided effect on the tumor growth. The method is inadequate for the determination of finer quantitative differences in the growth of tumors. (2) On the ninth day a drawing of the tumor was made. This drawing was compared with drawings of a series of other tumors of known weight, and thus the approximate weight of the tumor on the ninth day was determined. On the twelfth and thirteenth days further drawings of the tumors were made, and on the thirteenth day the tumors were removed and weighed, either each tumor individually or in smaller groups of tumors whose weight on the ninth day had been determined. On this day the increase in weight expressed in per cent. of the original weight during the period of treatment was ascertained. Before stating the effect of the various substances, it will be necessary to inquire into the degree of accuracy of our methods.

TABLE I.

Group No.	No. of tumors.	Calculated weight.	Actual weight.	Per cent.	
				+	−
1	21	6,525 gm.	5,717 gm. (+14%)	373%	331%
2	19	3,545 gm.	3,951 gm. (−11.4%)	17%	225%
3	10	2,550 gm.	2,575 gm. (> 1%)	26%	49%
4	15	2,910 gm.	3,377 gm. (14%)	52%	216%
		15,530 gm.	15,620 gm.	+468%	−821%
		−0.57%		−353%	

Average 5.8%

The first step in our second method consists in the calculation of the weight of the tumor on the ninth day on the basis of our drawings with the aid of weighed models. We investigated the accuracy of this determination by first calculating on several occasions the weight of a certain number of tumors nine days old by means of

drawings and then immediately removing the tumors and weighing them. We compared the weight of sixty-five tumors obtained first through calculation on the basis of drawings and models and subsequently through actual weighing. We arranged these into four groups according to the period in the course of our experiments at which the tumors were removed; in the first group are put together all those tumors removed in the beginning, at a time when the number of models at our disposal was still relatively small; in the fourth group those removed towards the latter part of our work, when the number of models had increased considerably.

In table I we find in the first column the weight calculated with the aid of drawings and models; in the second column the actual weight as determined with the aid of the balance. There is in the individual groups a maximum deviation of 14 per cent. in both directions, and if we add all the tumors there is found to be a little more than 0.5 per cent. error in the determination of weight by means of drawings and models. If we leave out of consideration the question of weight of the individual tumors and consider merely the means of the errors expressed in percentage of the actual weight, we find that on the average the weight of the individual tumors was underestimated. If we consider the fact that we examined usually two series of tumors at the same time and that therefore the error in both series which we wished to analyze was probably approximately of the same order and that the differences in weight which we produced experimentally were of a considerably higher order than the error of the method, and furthermore that all the experiments we made with this method were confirmatory of each other, we may conclude that the second method used is adequate for our purposes.

The reliability of the first method (palpation) we can test by determining in the same experiments the effect of the treatment by both methods. We first determine the increase in size by palpation and then the increase in weight by the second method. In weighing in a number of experiments each individual tumor, after its relative growth had previously been determined by palpation, and drawing and comparing its size on the thirteenth day with its original size on the ninth day, when the injections were started, we

could approximately come to a decision as to the accuracy of the first method.

TABLE II.

Comparison of Percentage of Growth of Tumors by Weighing and by Palpation.

	Tumors which by means of palpation had been diagnosed as retarded in growth.		Tumors which by means of palpation had been diagnosed as growing normally.	
	No. of mice.	Per cent. of growth (found through weighing).	No. of mice.	Per cent. of growth (found through weighing).
Series A II.				
Colloidal copper controls..	21	26%	14	70%
			In two cases we were uncertain, and both gave less than the average growth.	
Immunized mice.....	3	3%	17	102%
Series C II.				
Normal uninjected controls				
Class d.....	4	47%	11	203%
	In two cases we were uncertain whether retarded or not			
Class f.....	7	134%	28	294%
	In five cases we were uncertain whether retarded or not			
Mice injected with casein				
Class a.....	13	33%	10	97%
Class c.....	8	56%	8	163%
Class d.....	6	117%	4	236%
Class f.....	3	135%	4	291%
Mice injected with aurol				
Class a.....	12	68%	11	132%
Class c.....	4	12%	9	198%
Class d.....	3	−84%	8	202%
Class f.....	2	−54%	1	217%

Table II gives the results of these comparisons. We separated in each group those tumors which by palpation were found to show retarded growth from those showing normal growth, and determined in each the average increase in weight. The differences in actual weight between the two kinds of tumors are marked; and the results obtained by both methods agree, if we consider the various groups of tumors. In individual tumors there may occasionally be found a wrong diagnosis made by means of the first method.

In almost all cases the differences in actual weight between the tumors diagnosed as retarded in growth and growing normally are

more than 100 per cent. We have to consider that we must in each case compare tumors of the same class, inasmuch as tumors grow, as a rule, more rapidly from the ninth to the thirteenth day, the smaller they were on the ninth day; therefore, the tumors of class f grow more rapidly than tumors of class d; tumors of class d grow more rapidly than tumors of class c, and tumors of class c grow more rapidly than tumors of class a; and that therefore in class f a relative increase is regarded as retarded growth which in class a would have been considered as marked growth.

In other experiments we did not weigh each individual tumor, but certain groups of tumors, those tumors being in each case grouped together which had a similar weight on the ninth day. In this case we could less definitely determine how far the results obtained by both methods agreed, but, as far as could be judged, the results agreed fairly well.

In order to eliminate as much as possible the subjective factor in the first method, two persons made in a certain number of cases parallel experiments, using palpation and drawing as a means of determining the growth of the tumors; both persons arrived independently at the same conclusions. Furthermore, as far as the principal problems are concerned, we used both methods and the results obtained by both methods were on the whole confirmatory of each other. We may therefore conclude that the first method is sufficiently accurate to permit us to determine in a general, but not in an exact, quantitative way, whether or not a certain experimental interference had a retarding influence on the tumor growth. In a number of our earlier experiments we used, however, the first method exclusively, and we shall distinguish between results obtained by both methods and those obtained only by the first method.

We examined by these methods and within the time interval stated the action of various classes of substances, distilled water, a number of inorganic salts, a number of inorganic colloidal substances, various organic colloidal and non-colloidal substances, and especially hirudin alone and in combination with some other substances. We limited the treatment in the large majority of cases to four intravenous injections given on four consecutive days, because it was necessary to treat all animals in a comparable way. In ex-

periments on which we shall report in a further communication the four intravenous injections were preceded by a preliminary set of four intravenous injections. It is often difficult to give more than eight intravenous injections to a mouse, and we used, therefore, four injections as the standard for comparison.

In tables III, IV, V, and VI, those of our experiments in which the first method was used are grouped together. We determined first the lethal dose of each substance to be used. The quantity given varied somewhat in the case of the different substances. It was sometimes half the lethal dose, occasionally less, and in the majority of the cases somewhat more than one half the lethal dose. In the second column of our tables the lethal doses are represented, in the third column the dose given in our experiments. The fourth column gives the coefficient of safety; namely, the relation between the lethal and experimental dose. We used two criteria in judging the action of the various substances.

(1) One day after the first injection we examined the tumors, making incisions into them. Some substances exerted a distinct effect on the tumor after one injection. In columns 5 to 9 we gave the result in cases in which these examinations were made. Such examinations one day after the injection were not made in all the substances. We called "much affected" tumors those in which on the tenth day a large part of the growth was found to be necrotic and softened and sometimes hemorrhagic in the central portion. Some living tumor tissue may still be found in the periphery. In tumors which are designated as "partly affected" there is still a slight softening in the center of the tumor, but at least one half of the peripheral portion is not affected. In tumors "slightly affected" there is a very small area of softening in the central portion of the tumor, but most of the tumor appears normal.

(2) In columns 10, 11, and 12, we give the result of the effect of four injections on tumor growth, the figures showing the number of tumor mice observed. "Retarded growth" shows those tumors which grew distinctly less than the average tumor of a certain size. A certain number of tumors became distinctly smaller under the influence of the treatment. They are marked "retrogressing." It happens, of course, occasionally that a tumor retrogresses spon-

taneously, especially a tumor of small size; but this is on the whole not a usual occurrence. Only those mice were considered which lived through the whole period of the experiment, while those, for instance, which died on the fourth day were not included.

A number of the substances were obtained from manufacturers; others were prepared in our laboratory by Professor E. H. Keiser. Colloidal copper and platinum were prepared in accordance with Bredig's method.[2] Various organic substances were prepared according to the ordinary methods of biological chemistry.[3]

If we now consider the results in table III, we find that intravenous injection of distilled water did not retard the growth of tumors, neither did one injection cause any noticeable changes in the tumors. Two sulphur preparations (milk of sulphur, and hydrochloric acid plus sodium thiosulphate) were examined after one injection. No effect was noted. Various inorganic salts (copper nitrate, copper ammonium sulphate, mercury bichloride, gold sodium chloride (Merck), sodium tartrate, lanthanum nitrate) did not show any

[2] Some other substances were prepared in the following manner:

Copper Casein B.—Add a concentrated solution of $CuSO_4$ to an equal amount of a saturated solution of casein (dissolved in NaOH). Dissolve the precipitate in NH_4OH and precipitate with absolute alcohol.

Copper Casein C.—Dissolve copper tartrate in NaOH and add to an equal quantity of saturated casein solution (dissolved in NaOH). Precipitate in absolute alcohol.

$H_2S + NaNO_2$.—Pass H_2S through a moderately strong $NaNO_2$ solution; this gives a yellow precipitate. Dilute with H_2O. After standing for twenty-four hours the precipitate is dissolved.

Sulphur.—Colloidal (chemically prepared). Heat flowers of sulphur with NaOH and a little water; boil and pass steam through a condenser. This gives a milky liquid with slight reddish color.

Selenium.—Colloidal (chemically prepared). Dissolve the selenium by boiling with concentrated HNO_3, and evaporate this to dryness. Heat and sublime the SeO_2. Dissolve the crystals in H_2O and then run in H_2S. This gives an orange colored liquid.

Casein and Sulphur.—Dissolve 10 gm. of casein (purified) in 600 c.c. of distilled water to which 20 c.c. of normal NaOH have been added. Pass a stream of H_2S through this for from twelve to twenty-four hours, until saturated with H_2S. This gives an acid reaction to litmus. Then draw air through until the H_2S is no longer perceptible.

[3] Protamin was prepared according to Kossel's method from dried salmon milt, which we obtained through the kindness of Professor Alonzo E. Taylor, of the University of Pennsylvania.

TABLE III.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection.				Several injections.		
				Much affected.	Partly affected.	Slightly affected.	Normal.	Retarded growth.	Retrogressing.	Normal growth.
Copper nitrate m/250	0.6 c.c.	0.4 c.c.	1 : 0.66	—	—	—	—	2	—	25
Copper ammonium sulphate 0.7%	—	0.3 c.c.	—	—	—	—	—	1	—	10
		0.21 mg.								
Mercury bichloride m/400	0.4 c.c.	0.25 c.c.	1 : 0.62	—	—	—	—	—	—	5
Lanthanum nitrate	4.0 mg.	2.5 mg.	1 : 0.62	—	—	—	—	—	—	3
Sodium tartrate 3⅛%	0.5 c.c.	0.4 c.c.								
	17.0 mg.	14.0 mg.	1 : 0.8	—	—	—	—	—	—	15
Gold potassium cyanide (Merck)	0.15 mg.	0.05 mg.	1 : 0.33	—	—	—	—	5	—	17
Gold sodium chloride (Merck)	0.8 mg.	0.4 mg.	1 : 0.5	—	—	—	—	1	—	20
Distilled water	—	1.0 c.c.	—	—	—	—	3	2	—	19
Milk of sulphur	0.75 c.c.	0.37 mg.	1 : 0.5–							
		0.16 c.c.	0.25	—	—	—	3	—	—	—
Hydrochloric acid + sodium thiosulphate	0.75 c.c.	0.5 mg.	1 : 0.66–							
		0.25 c.c.	0.33	—	—	—	7	—	—	—

TABLE IV.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection.				Several injections.		
				Much affected. (1)	Partly affected. (2)	Slightly affected. (3)	Normal.	Retarded growth.	Retrogressing.	Normal growth.
Colloidal copper	2.5 c.c. +	1 c.c.	1 : 0.4–	—	—	1	18	371	—	277
Colloidal copper hydrate (Mulford)	0.1 c.c.	0.05 c.c.	1 : 0.5	—	—	—	—	2	1	20
Colloidal copper (Heyden) 0.3%	0.7–0.4 c.c.	0.25 c.c.								
	2.1–1.2 mg.	0.75 mg.	1 : 0.35–0.62	15	—	1	2	10	—	4
Colloidal platinum	2.5 c.c. +	1.0 c.c.	1 : 0.4–	—	—	—	—	12	—	3
Colloidal sulphur (electrolytic)	—	1.0 c.c.	—	—	—	—	—	3	—	7
Colloidal sulphur (chemical)	—	1.0 c.c.	—	—	—	—	—	1	—	10
Colloidal selenium (Mulford)	0.1 c.c.									
	0.025 c.c.	0.0125 c.c.	1 : 0.125–0.5	—	—	—	—	6	—	15
Colloidal selenium (prepared by us chemically)	—	1.0 c.c.	—	—	—	—	—	—	—	11
Aurol (Hille) 15%	0.7 c.c.	0.5 c.c.	1 : 0.72	—	—	—	—	14	7	29
Copper casein A	1.2 mg.	1.0 mg.	1 : 0.83	—	—	—	—	8	—	3
Copper casein B	1.2 mg.	1.0 mg.	1 : 0.83	—	—	—	—	—	—	9
Copper casein C	12 mg.	10.0 mg.	1 : 0.83	—	—	—	—	11	—	—

noticeable inhibiting effect on tumor growth. Although the number of tumor mice treated was not great in the case of each individual substance, the number is sufficiently large (eighty-three) if we add animals used in the various experiments. We may therefore conclude that inorganic salts injected under the conditions of our experiments had not a sufficiently marked retarding influence on tumor growth to be accessible to determination by our first method. This does not exclude the possibility that through our second method a slight influence might be found. Only in the case of gold potassium cyanide was there possibly a slightly retarding influence present, but if present at all, it certainly was not marked.

In table IV some inorganic colloidal substances and combinations between copper and casein are considered. Besides the colloidal copper prepared by us electrolytically in accordance with Bredig's method we used a preparation of colloidal copper made by the H. K. Mulford Company, and one by Heyden (Dresden). The exact composition of these two preparations is not known to us. Colloidal platinum we prepared ourselves in accordance with Bredig's method. Colloidal sulphur we prepared either chemically or by the electrolytic method of Svedberg. Aurol (Hille) is a preparation of colloidal gold which was sent us by the Vitochemical Laboratories, Chicago.[4] Professor Keiser prepared several combinations of copper and casein (B and C).

The results are as follows: Colloidal copper prepared electrolytically, colloidal copper (Heyden), colloidal platinum, aurol (Hille), and two of the copper casein preparations had a distinct retarding influence on tumor growth; while the colloidal copper hydrate (Mulford), colloidal sulphur prepared chemically, colloidal selenium prepared by us chemically, and one copper casein preparation (B) were without decided influence. It is possible that the copper casein preparation B had undergone secondary changes at the time we used it and that this fact may account for the negative results. In the case of the electrolytically prepared sulphur and colloidal selenium (Mulford) there was perhaps a slight effect present, but it was certainly not marked. It is possible that small quantities of colloidal

[4] The H. K. Mulford Co. and the Vitochemical Laboratories kindly put their preparations at our disposal for experimental purposes.

platinum were admixed to the solution of electrolytically prepared sulphur and that this may account for a slight effect which was possibly present.

Our method does not permit us to differentiate quantitatively between the efficacy of the various active substances. We observed a temporary retrogression during the period of injection in a few animals treated with aurol; but the number of retrogressing tumors in this case is too small to be of much significance. On the whole, however, the difference in the efficacy of the various preparations does not seem to be striking. Two preparations were tested as to their early effect on the tumor after one injection. Our own preparation of colloidal copper was without effect in this respect, while the Heyden preparation of colloidal copper had a marked effect.

We may then conclude that in contradistinction to the inorganic salts of molecular dispers character, which are without marked effect, certain colloidal solutions of heavy metals (copper, platinum, gold) are effective, inasmuch as they retard definitely the growth of a number of tumors of injected animals; of a similar activity are also combinations of copper salts and casein. However, according to the method of preparation of some of these substances their efficacy differs. None of these substances has a markedly destructive action on mouse tumors. In the majority of cases the effect is transitory and does not lead to a cure.

In table V the results of the injections of various organic substances are represented. We used various albuminous substances, as well as starch and lecithin, as representative of the carbohydrates and lipoids. In addition we used horse serum; tuberculin we used as a representative of bacterial toxins. Ethylhydrocuprein was successfully used by Morgenroth[5] in the treatment of pneumococcus infections. Ergamin is a substance producing symptoms somewhat resembling anaphylactic shock. Of all these substances only casein and nucleoproteid preparations had a sufficiently retarding effect on tumor growth to be demonstrable by our method. And the effects of these substances seemed to be on the whole of a similar order to those produced by the colloidal metals. They differ, however, from

[5] We are indebted to Professor Morgenroth and the manufacturers for the material at our disposal.

TABLE V.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection.				Several injections.		
				Much affected.	Partly affected.	Slightly affected.	Normal.	Retarded growth.	Retrogression.	Normal growth.
Ethylhydrocuprein 0.375% ...	0.4 c.c.	0.25 c.c.								
	1.5 mg.	0.94 mg.	1 : 0.62	—	—	—	—	—	—	4
Ergamin (Burroughs and Wellcome).....	—	1.0 mg.	—	—	—	—	—	—	—	6
Starch 5% suspension.....	1.0 c.c. +	0.75 c.c.	1 : 0.75 +	—	—	—	11	—	—	6
Lecithin 0.1% (Merck ovo)...	0.75 c.c.	0.5 c.c.								
	0.75 mg.	0.5 mg.	1 : 0.66	—	—	—	6	—	—	6
Pepton 5%	1.5–1.25 c.c.	0.6 c.c.								
	0.1–0.075 mg.	0.03 mg.	1 : 0.4–0.5	—	—	—	13	101	—	21
Protamin 0.1% .	0.7 c.c.	0.5 c.c.								
	0.7 mg.	0.5 mg.	1 : 0.72	—	—	—	10	—	—	10
Serum globulin 15%.....	2.0–1.5 c.c.	1.0 c.c.								
	0.3–0.22 gm.	0.15 mg.	1 : 0.5	—	—	—	23	—	—	9
Ovalbumin saturated	1.5–1.0 c.c.	0.75 c.c.								
	0.37–0.25 gm.	0.18 gm.	1 : 0.5–0.75	—	—	—	102			23
Tuberculin (AT) 50%.....	0.65–0.2 c.c.	0.5–0.15 c.c.	1 : 0.8–0.75	—	—	—	14	—	—	9
Horse serum.....	1.5 c.c.	1.0 c.c.	1 : 0.66	—	—	—	20	—	—	4
Casein 1⅔%.....	2.0 c.c.	1.0 c.c.								
	32.0 mg.	16.0 mg.	1 : 0.5	46	6	20	22	68	—	54
Casein 1⅔% + sulphur.....	2.0 c.c.	1.0 c.c.								
	32.0 mg.	16.0 mg.	1 : 0.5	66	15	23	3	34	—	16
Nucleoproteid (lymph gland of cattle) 15%....	0.75–0.25 c.c.	0.5–0.15 c.c.								
	113.0–38.0 mg.	76.0–22.0 mg.	1 : 0.66	18	1	4	28	7	—	5
Nucleoproteid (kidney of cattle) 15%.....	0.25–0.15 c.c.	0.2–0.1 c.c.								
	38.0–22.0 mg.	30.0–15.0 mg.	1 : 0.8–0.66	10	2	5	13	17		9

the electrolytically prepared colloidal copper through the edema or hemorrhages which they cause and which is noticeable twenty-four hours after the first injection in many tumors of the injected mice. Here again casein and nucleoproteid were the only active substances, while all the other substances in table V were without direct effect on the tumors.

In table VI the effect of hirudin and various combinations of hirudin and other substances is given. On the whole, hirudin is, as far as the number of affected tumors is concerned, not more effective than colloidal copper. It differs, however, from colloidal copper in

TABLE VI.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection.				Several injections.		
				Much affected.	Partly affected.	Slightly affected.	Normal.	Retarded growth.	Retrogression.	Normal growth.
Hirudin 0.25% solution	1.5–0.1 c.c.	0.05–1.0 c.c.	1 : 0.5–0.065							
	3.7–0.25 mg.	2.5–0.125 mg.		11	—	—	—	114	70	102
Hirudin + nucleoproteid	—	Usual dose of hirudin + usual dose of nucleoproteid		10	—	—	—	1	11	2
Colloidal copper + hirudin	—	Colloidal copper 0.5–0.25 c.c. Hirudin 0.125–0.25 mg.		—	—	—	—	11	24	6
Different one each day: colloidal copper, casein, hirudin, nucleoproteid	—	Usual doses		—	—	—	—	34	—	12
Colloidal copper, intravenously. Hirudin, subcutaneously	—	Colloidal copper 1.0 c.c. Hirudin 0.80 mg.		—	—	—	—	7	—	6
Hirudin, subcutaneously 0.25%		0.25 c.c. 0.8 mg.		—	—	—	—	2	—	9

two respects: (1) the number of tumors retrogressing under the influence of hirudin is considerably greater; and (2) one single injection has a marked effect on the tumor, causing hemorrhage and edema. As we saw previously, colloidal copper did not have such an effect. The retrogression of the tumors is usually not a permanent one; but after temporary cessation of the injections the tumors begin in the majority of cases sooner or later to grow again; a certain number of the tumors, however, retrogress definitely. Hirudin

given subcutaneously was without a marked effect. It is, however, to be noted that the efficacy of several samples of hirudin differed and that the results obtained at different periods were not equally marked. More effective than hirudin alone was a combination of hirudin and either nucleoproteid or colloidal copper, both substances being given at the same time. In combination with nucleoproteid the number of retrogressions was considerably greater than in cases in which hirudin alone was used. It is, however, to be considered that at that time the hirudin which we used was stronger than some later samples that we received. A combination of colloidal copper and hirudin was also effective. The number of retrogressing tumors was here very great, much greater than with either hirudin or colloidal copper alone. This combination is also highly toxic and a large number of the tumor mice injected died. If the hirudin was given subcutaneously instead of intravenously, while the colloidal copper was given intravenously, the effect was merely that of the colloidal copper. Hirudin administered subcutaneously was therefore again ineffective.

As we shall see later, processes of immunization take place in the course of a series of injections. It was therefore of interest to test the effect of a combination of four different substances, a new substance being given on each successive day. Colloidal copper, casein, hirudin, and nucleoproteid were thus alternately administered. There was, perhaps, a somewhat stronger effect than in cases in which each of these substances was given alone for four successive days, but the difference was not marked. This result points to the conclusion that one single injection of a certain substance does not lead to the production of a noticeable degree of immunity.

In addition to these experiments carried out by means of the first method, we carried out a series of experiments with the second method, in order to obtain results of a more quantitative character.

All the tumors treated in an experiment in the same manner represented a series. Inasmuch as the percentage increase of tumors in weight or volume depends on their weight at the time when the experiment was started, we had to subdivide a series in different classes, according to the weight of the tumors on the ninth day. In

all experiments the classes were as follows: class a = tumors weighing 360 milligrams or more; class c = tumors weighing between 230 and 360 milligrams; class d = tumors weighing between 130 and 230 milligrams; class f = tumors weighing between 25 and 130 milligrams.

In cases in which we were unable to weigh each tumor at the end of the experiment, we divided the various classes into smaller groups, according to the weight of the tumors on the ninth day, and all the tumors of one group were weighed together. In table VII the results obtained by the second method are given.

In the first column we find the number of mice used in testing each substance. In the case of pepton and ovalbumin we referred separately to the individual experiments made with each of these substances. In addition we give a summary of the three experiments done with each of these two substances. In the third experiment we give two sets of figures; in one set all the tumors were included; in the corrected set we omitted from consideration the various retrogressing tumors. This seemed to be a fairer way of calculation, because in this experiment there were also a number of retrogressions in the control tumors. In all our tables only the tumors of such animals were included as lived to the end of the experiment. All animals that died before that time were omitted. The last four columns of the table (a, c, d, f) give the percentage increase in weight in the different classes of tumors arranged in accordance with their weight nine days after inoculation; a represents the largest, f the smallest tumors. Columns 2, 3, 4, and 5 (α, β, γ, δ) give the average percentage increase of the tumors of a series. We describe in the following paper[6] on "Immunization against substances inhibiting tumor growth" the methods used by us for the determination of these averages.

In the first line in table VII the percentage increase of normal control mice is given. The second line gives the corresponding figures for mice injected with colloidal copper. We recognize clearly the depressing effect of colloidal copper on tumor growth. While the normal control tumors gain 170 to 180 per cent. in weight in four days, in the tumors of the injected animals, including those in

[6] Fleisher, M. S., Vera, M., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 522.

TABLE VII.

	No. of mice.	α	β	γ	δ	a	c	d	f
Normal controls	262	177%	192%	174%	180%	117%	140%	181%	251%
Colloidal copper	300	103%	107%	110%	107%	81%	81%	123%	155%
Aurol	50	111%	112%	97%	106%	[23]98%[7]	[13]137%	[11]141%	[8]11%
Hirudin	69	139%	150%	147%	145%	———	[7]126%	[27]150%	[35]166%
Casein	56	84%	112%	138%	111%	[23]61%	[15]104%	[10]158%	[7]228%
Pepton 1	42	111%	154%	131%	121%	[3]60%	[5]145%	[13]132%	[21]185%
Pepton 2	52	191%	192%	183%	189%	[3]135%	[4]195%	[17]220%	[28]181%
Pepton 3	28							[14]157%	[14]108%
Corrected, omitting retrogressing tumors similar to those in controls	22							[12]177%	[10]213%
Pepton. Summary	122	152%	173%	157%	155%	94%	170%	169%	158%
Pepton. Summary. Corrected figures.	116							176%	193%
Ovalbumen 1	39	185%	174%	176%	177%	[1]120%	[2]236%	[14]182%	[22]166%
Ovalbumen 2	48	166%	192%	159%	172%	[4]99%	[6]123%	[15]182%	[23]233%
Ovalbumen 3	53	145%	161%	157%	154%	[8]99%	[14]134%	[14]118%	[17]277%
Corrected, omitting retrogressing tumors	46	160%	205%	191%	185%	[7]120%	[13]145%	[12]176%	[14]325%
Ovalbumen. Summary	140	165%	176%	164%	168%	106%	164%	161%	225%
Ovalbumen. Summary. Corrected figures	133	170%	191%	175%	178%	113%	169%	180%	241%

[7] The small figures at the upper left side of some of the percentage figures in the last four columns indicate the number of mice used in the various experiments.

which no effect was noticeable, as well as those which were influenced by the colloidal copper, the increase was from 100 to 110 per cent.

As will be seen from the tables, our figures in the case of colloidal copper are based on the examination of a large number of mice.

Aurol is effective, but probably less so than colloidal copper. In this experiment some of the smaller tumors retrogressed and the figure for f is therefore very low. The low percentage increase in group f reduces considerably the average increase of the tumors treated with aurol. Inasmuch as the number of mice in class f was very small, we must not attach too much importance to this figure. In the other classes the reduction in percentage increase in weight was considerably less than in colloidal copper. In the case of aurol we noticed a dark bluish grey discoloration of the tumors, liver, and occasionally the spleen, after four injections had been given. Apparently this discoloration was due to a deposit of foreign material in capsule and stroma.[8]

Hirudin also had a distinctly inhibiting effect. The full effect can, however, not be appreciated from a study of our figures, as there was much coagulated blood and marked edema in the tumors of the injected mice. The effect of hirudin is therefore more marked than is indicated by the figures of our table. Besides, a number of these tumors are usually found to retrogress when the observation of the tumors of mice injected with hirudin is prolonged.

Casein is also effective, but perhaps not quite so effective as colloidal copper. Here the average increase determined according to the methods is especially low because the number of the large tumors was great and their growth was unusually retarded.

While casein served as a representative of the albuminous substances which we had found effective in employing the first method of measurement, we used pepton and ovalbumin as representatives of those proteids which the first method showed to be ineffective, or less effective. With each of these two substances we made three experiments. In the first experiment the mortality among the in-

[8] We intend to examine the character of the deposit more closely as soon as possible. On fixed and stained specimens the cause of the discoloration could not be determined.

jected mice had been very great, about one half of all the injected mice dying after the first injection. The remaining mice also were somewhat affected. The mice injected with pepton showed very much retarded tumor growth. The effect was here similar to colloidal copper. The mice injected with ovalbumin behaved differently in different classes. In this experiment the first dose of the substances given had evidently been too strong and the general health of the mice may have suffered in consequence. We may state that neither after injection of colloidal copper nor of casein does the general condition of the mice seem to suffer.

In the second experiment an effect on the part of pepton as well as ovalbumin was either absent or very small. A slight reduction in class f must not be overestimated, inasmuch as in class f spontaneous retrogressions are apt to occur. In the third experiment there occurred in the control experiments a number of spontaneous retrogressions of tumors. Retrogression of tumors in the animals injected with pepton or ovalbumin must therefore in the main not be attributed to the action of these substances, and the mice with retrogressing tumors should be entirely omitted, in order to obtain a fair estimate of the action of these substances. With these corrected figures ovalbumin was without effect in the third experiment, while the original figures indicated some effect of ovalbumin, which was, however, considerably less than casein. With pepton only tumors of classes d and f were used in this experiment. There was in this case some effect in class f noticeable in using either of the two figures. The corrected figures of class d were approximately the figures of normal mice, while the uncorrected figures were similar to those of casein. If we consider the summary of the three experiments we find that pepton has decidedly less effect on the tumors than casein, although it has some effect, while ovalbumin is almost without effect. These experiments make it probable, therefore, that ovalbumin and pepton are decidedly less effective than casein, although they may have some effect, especially pepton; and they confirm therefore, on the whole, the conclusions based on the first method. We believe, however, that further experiments are necessary to make our conclusion concerning the different efficacy of various proteids definite.

While in the case of most substances which we used we studied the effect on the growth between the ninth and thirteenth day after the inoculation of the tumor, we determined in the case of colloidal copper and of hirudin in addition the effect of injections given from the second to the sixth day after inoculation. We found that the tumors of animals treated in this way were on the ninth day after inoculation as large as tumors of animals which had not been injected. It seems, therefore, that injections given at an early date are without a noticeable effect on tumor growth. This is perhaps due to the fact that at the early period the vascularization of the tumor has not yet progressed as far as later, and perhaps also to the lesser interference with the circulation through pressure at this early period. If, however, the tumors have reached a certain development, such as is found between the ninth and twelfth days after inoculation, they are much more accessible to the action of colloidal copper.

TABLE VIII.

Decrease of Percentage Increase in Weight under the Influence of Colloidal Copper in the Different Classes.

Group.	Tumors. Control mice.	Increase in weight of tumors in mice injected with copper.
a	100 %	69 %
c	100 %	58 %
d	100 %	68 %
f	100 %	62 %

It was of interest to determine whether there was any difference between small and large tumors in the degree with which they are influenced by the injection of colloidal copper. In table VIII the tumors are arranged in classes, a, c, d, f, according to their size. If we take 100 per cent. as the standard of the average increase in weight of the various classes of control tumors, the tumors in the various classes of injected mice lose approximately in the same proportion under the influence of colloidal copper. Tumors of different size are therefore relatively influenced in a similar manner through the injections of colloidal copper. While the relative loss in all classes is approximately the same, the absolute loss is of course greater, the greater the percentage increase in weight of the different classes in normal control mice.

SUMMARY.

We have described the methods used in determining the influence of certain substances on tumor growth, and we measured approximately the degree of reliability of the quantitative method used. We examined with these methods various classes of substances,—distilled water, a number of inorganic salts, inorganic colloidal substances, various organic colloidal and non-colloidal substances, especially various proteids, tuberculin and hirudin alone as well as in combination with other substances. Distilled water, various inorganic sulphur preparations, and various inorganic salts did not show an inhibiting effect on tumor growth sufficient to be detected by means of our first method. Only in the case of gold potassium cyanide was there possibly a slightly retarding influence present. On the other hand, certain colloidal solutions of heavy metals (copper, platinum, gold) retard the growth of a number of tumors of injected animals. Certain combinations of copper salts and casein act in a similar manner. Of the organic substances used, casein, nucleoproteid, and hirudin were active, while the other proteids tested, as well as various other organic substances and tuberculin and lecithin, seemed to be either without effect or weaker than the other substances mentioned as retarding the tumor growth. Hirudin was active and caused in addition to its inhibiting influence the retrogression of a certain number of tumors. Especially active was a combination of hirudin with colloidal copper and of hirudin with nucleoproteid.

One single injection of casein or nucleoproteid, or of the Heyden preparation of colloidal copper, leads to a more or less marked edematous condition of a certain number of tumors, while hirudin caused in addition, in many cases, marked hemorrhages in or around the tumors. Other substances which we tested did not show this effect, although their inhibiting action on tumor growth may have been equally strong. Very young tumors (two to six days old) are not retarded in their growth through injection of colloidal copper or hirudin, while nine to thirteen days old tumors are, independently of their size on the ninth day, inhibited in approximately the same relative degree; absolutely, however, the more rapidly growing smaller tumors are more markedly inhibited than the normally more slowly growing larger tumors.

IMMUNIZATION AGAINST THE ACTION OF SUBSTANCES INHIBITING TUMOR GROWTH.*

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Theoretical as well as practical considerations suggested to us the line of investigations that is reported in this communication. One of us[1] has formerly shown that through the action of heat or chemical injurious influences to which the tumor cells are subjected before transplantation it is possible to decrease the virulence of the resulting tumors. It was of interest to inquire to what extent this peculiarity of growth might be inherited by new generations of tumor cells, and one of us[2] investigated, therefore, the resistance to heat of particles of tumor which had developed in animals from tumor particles exposed to a certain degree of heat before transplantation.

We found with the method used at that time that neither a summation of the effects of successive exposures to heating nor an immunity against the effects of heat can be demonstrated under these conditions. We also found that after transplantations of particles of tumors which developed after previous heating, the new tumors grew as well as control tumors obtained through transplantation of particles from previously unheated tumors. Notwithstanding these negative results the problem seemed to us of sufficient importance to justify further attempts on the basis of the

* Received for publication, July 16, 1914.

[1] Loeb, L., *Virchows Arch. f. path. Anat.*, 1903, clxxii, 345; *Am. Med.*, 1905, x, 265.

[2] Corson-White, E. P., and Loeb, L., *Centralbl. f. Bacteriol., 1te Abt., Orig.*, 1910, lvi, 325.

results communicated in the preceding paper.[3] We have shown that a number of different substances exert a definite inhibiting action on the growth of mouse tumors. The opportunity presented itself to investigate whether an immunity or increased resistance[4] can be produced through a series of preliminary injections, and whether such an immunization depends on processes called forth in some organ or tissue of the injected animal or in the tumor cells proper.

There was a further reason for undertaking these investigations. In a certain number of cases we observed in patients suffering from cancer that at first the intravenous injections of colloidal copper had an undoubted effect on the tumor, causing diminution in the size of the ulcerated area and partial shrinking of the tumor or an arrest in the progress of the disease which had been rapid before. But these effects were temporary and after a certain time the disease began again to progress. This observation could be explained by the assumption of processes of immunity against the injected substance taking place either in the injected organism or in the tumor cells, a possibility which we had indeed considered from the beginning of our experiments.

In order to test the existence of such processes of immunity we proceeded in the following manner: We injected intravenously a series of animals with colloidal copper from the ninth to the thirteenth day after transplantation of the tumor, and we determined the growth of these tumors. In a second series we injected animals from the second to the fifth day after inoculation with colloidal copper and again from the ninth to the thirteenth day. We had

[3] Loeb, L., and Fleisher, M. S., *Jour. Am. Med. Assn.*, 1913, lx, 1857. In a different manner Marie, Clunet, and Raulot-Lapointe recently observed a hereditary transmission of a characteristic acquired by sarcomatous cells of the mouse under the long continued influence of Roentgen rays. Under these conditions giant cells, which were absent in the original sarcoma, began to develop and this peculiarity was also noticeable in the following generations of transplanted tumor, although these had not been exposed to the Roentgen rays. (Marie, P., Clunet, J., and Raulot-Lapointe, G., *Bull. de l'Assoc. franç. p. l'étude du cancer*, 1911, iii, 166; abstracted in *Ztschr. f. Krebsforsch.*, 1913, xii, 93.)

[4] We do not attempt to distinguish between partial or total immunity and increased resistance. There do not exist as far as we are aware any sharp criteria which permit a differentiation between two conditions corresponding to these two terms.

previously found that injections of colloidal copper given from the second to the fifth day had no noticeable influence on the tumor growth. Inoculated animals receiving these early injections showed on the ninth day tumors of approximately the same size as animals not receiving these early injections. It was now of interest to inquire whether the early injections which failed to have an inhibiting effect might to a certain extent immunize the animals against the effect of the second set of injections given from the ninth to the thirteenth day. If this should be the case, the injection given from the ninth to the thirteenth day should have less of an inhibiting effect in previously injected animals than in control animals which had not had the preliminary set of injections. We kept another set of control animals which did not receive any injections. If an immunizing effect of the first set of injections should be found to exist, it was furthermore necessary to inquire whether the processes of immunity were restricted to reactive changes in injected animals or in the tumor cells.

This was determined in the following way: A series of animals received, after inoculation with the tumor, two sets of injections of colloidal copper; namely, from the second to the sixth and from the ninth to the thirteenth days after inoculation. Two days after the last injection of colloidal copper, several tumors were extirpated and used for inoculation in a new series of mice. Another series of mice was inoculated with tumor material from mice which had not previously received injections of colloidal copper. Especial care was taken that in both series tumors of approximately similar growth energy were used for transplantation. Both series of mice were then injected with colloidal copper from the ninth to the thirteenth day. If the tumor cells, derived from tumors which had grown in animals injected with colloidal copper, should prove decidedly more resistant towards the effect of the injections of colloidal copper, we may conclude that the tumor cells have been to some extent immunized against the effect of colloidal copper. As controls we kept another series of mice previously inoculated with tumor without injections. We further gave to one series of mice two sets of injections of hirudin, the first set from the second to the sixth day and the second from the ninth to the thirteenth day, and

to another series we gave only the second set of hirudin injections, in order to determine whether also in the case of hirudin an immunity could be produced through previous injections. Just as in the case of colloidal copper, injections of hirudin given from the second to the sixth day do not influence the development of tumors to any marked extent; on the ninth day such tumors are about as large as tumors of animals not previously injected.

In order to determine the effect of the injections we employed in a number of these experiments both methods, described by us in the preceding paper,[5] determining the growth of the tumors on the ninth day by palpation and drawing, and afterwards on the thirteenth day again determining the growth of the tumors by drawing and palpation and then removing the tumors and weighing them either individually or in groups of tumors which were so arranged that tumors united in the same group had about the same size on the ninth day. In addition we carried out other experiments in which we used merely the first method, in order to corroborate the results obtained by the more accurate second method. We made use of the first method in order to determine whether a partial immunity towards the effects of hirudin is located in the tumor cells and is transmitted to following cell generations in a similar manner as in the case of colloidal copper. We furthermore injected a series of animals with colloidal copper or hirudin before inoculation with the tumor in order to determine whether through the injections reactive processes are set up in the organism of the animal, independently of the processes in the tumor cells. Furthermore, by means of the first method we tested the specificity of the immunity produced, giving in the first set of injections, from the second to the sixth day, colloidal copper or hirudin, and in the second set, from the ninth to the thirteenth day, the substance not used in the first set of injections. In this way we could determine whether injections of colloidal copper immunize against the effect of hirudin and *vice versa*. We also tested in a similar manner whether injections of hirudin or colloidal copper, given in one generation of animals, immunize the tumor cells against the substance not used in the first generation in the following generation of animals after transplantation of the treated tumor.

[5] Fleisher, M. S., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 503.

TABLE I.

Control (uninjected) mice.			Mice injected with colloidal copper 9th–13th dy.			Mice injected with colloidal copper 2d–6th and 9th–13th dys.			Mice inoculated with tumor from mice previously injected with colloidal copper. Injected 9th–13th dy.		
	R	B		R	B		R	B		R	B
H	53 mice		A1	24 mice		A2	18 mice		A1	51 mice	
	α 174%	2 m.a. + 113%		α 63% ●	5 m.c. + 48%		α 110% ●	5 m.c. + 74%		α 142% ●	18 m.c. + 100%
	β 194%	10 m.c. + 143%		β 121% ●	9 m.d. + 102%		β 126% ●	7 m.d. + 123%		β 282% ●	12 m.d. + 182%
	γ 166%	18 m.d. + 147%		γ 85% x	10 m.f. + 110%		γ 128% x	6 m.f. + 172%		γ 178% x	21 m.f. + 451%
	δ 178%	23 m.f. + 259%		δ 96%			δ 120%			δ 201%	f.x. + 300%
D	83 mice		A2	34 mice		B1	60 mice				
	α 174%	4 m.a. + 96%		α 69% ●	6 m.c. + 40%		α 127%	12 m.a. + 97%			
	β 177%	8 m.c. + 144%		β 29% ●	5 m.d. + 52%		β 140%	26 m.c. + 121%			
	γ 153%	35 m.d. + 218%		γ 49% x	23 m.f. + 22%		γ 151%	14 m.d. + 171%			
	δ 168%	36 m.f. + 155%		δ 49%			δ 139%	8 m.f. + 217%			
B1	19 mice		B1	59 mice					B2	62 mice	
	α 164%	1 m.a. + 95%		α 104%	17 m.a. + 76%					α 139%	9 m.a. + 103%
	β 201%	6 m.c. + 116%		β 88%	30 m.c. + 67%					β 175%	22 m.c. + 106%
	γ 179%	6 m.d. + 192%		γ 127%	8 m.d. + 144%					γ 177%	18 m.d. + 231%
	δ 181%	6 m.f. + 312%		δ 106%	4 m.f. + 220%					δ 160%	13 m.f. + 269%
B2	39 mice		B2	59 mice							
	α 176%	10 m.a. + 156%		α 83%	10 m.a. + 52%						
	β 198%	6 m.c. + 155%		β 138%	14 m.c. + 103%						
	γ 187%	5 m.d. + 183%		γ 134%	12 m.d. + 171%						
	δ 187%	18 m.f. + 255%		δ 118%	13 m.f. + 211%						
C1	18 mice		C1	58 mice		C1	55 mice				
	α 138% ●	3 m.a. + 126%		α 125%	14 m.a. + 98%		α 209%	4 m.a. + 187%			
	β 143% ●	12 m.c. + 140%		β 150%	15 m.c. + 107%		β 230%	16 m.c. + 172%			
	γ 182% x	3 m.d. + 184%		γ 149%	14 m.a. + 172%		γ 223%	19 m.d. + 236%			
	δ 154%			δ 141%	15 m.f. + 218%		δ 221%	16 m.f. + 296%			

TABLE I.—*Concluded.*

Control (uninjected) mice.		Mice injected with colloidal copper 9th–13th dy.		Mice injected with colloidal copper 2d–6th and 9th–13th dys.		Mice inoculated with tumor from mice previously injected with colloidal copper. Injected 9th–13th dy.	
R	B	R	B	R	B	R	B
C2 50 mice		C2 66 mice				C2 68 mice	
α 220% ●	15 m.d. + 162%	α 113%	8 m.a. + 98%			α 204%	6 m.a. + 160%
β 237% ●	35 m.f. + 272%	β 117%	20 m.c. + 118%			β 232%	22 m.c. + 157%
γ 174% x		γ 114%	15 m.d. + 95%			γ 222%	22 m.d. + 274%
δ 210%		δ 115%	23 m.f. + 147%			δ 219%	18 m.f. + 296%
262 mice		300 mice		133 mice		181 mice	
α 177%	a + 117%	α 103%	a + 81%	α 163%	a + 142%	α 200%	a + 131%
β 192%	c + 140%	β 107%	c + 81%	β 165%	c + 122%	β 229%	c + 121%
γ 174%	d + 181%	γ 110%	d + 123%	γ 167%	d + 176%	γ 189%	d + 229%
δ 180%	f + 251%	δ 107%	f + 155%	δ 165%	f + 228%	δ 206%	f + 339%

α Percentage of increase on comparing the sum of the original weight of all tumors (calculated) with the sum of the end weight (weighed).

β Multiply the average percentage of increase of each class (a, c, d, f) by the number of mice in that class, add the figures thus obtained, and divide the sum by the total number of mice used in that series.

γ Average percentage of increase of the various classes; add the percentage increases in each class and divide by the number of classes.

δ Average of the three preceding averages.

The figures with the circle (●) are calculated from incomplete experiments, as one or more classes are missing.

The figures with the letter x are calculated from the figures shown in the various classes in which an average selected in accordance with the experimentally determined figures was substituted for the missing classes. They are in A1, column 4, calculated on a basis of corrected figures.

The series in each column which have the same mark, for instance A1 and B1, represent experiments done under approximately the same conditions and at the same time.

In column R are given the figures for the entire series; in column B are given the figures for the smaller classes (a, c, d, f) and in addition the number of mice (m.) in each class. For instance, 2 m.a. signifies that in a certain experiment there were 2 mice in class a.

We distinguished between the results obtained by means of a combination of both methods and those obtained merely by the first method. The results obtained by means of both methods, and in which the results of both methods corroborate each other, must necessarily be considered more secure than the results based merely on the first method. We shall therefore consider both sets of experiments separately. The experiments carried out by means of both the first and second methods are represented as far as the effects of colloidal copper are concerned in table I. Here is compared the percentage growth from the ninth to the thirteenth day after inoculation (1) of control mice not receiving any injections, (2) of mice injected from the ninth to the thirteenth day with colloidal copper, (3) of mice injected from the second to the sixth and from the ninth to the thirteenth days with colloidal copper, and (4) of mice of the second generation which had been inoculated with pieces of tumor from mice of the first generation which had been injected with colloidal copper from the second to the sixth and again from the ninth to the thirteenth days after tumor inoculation. These mice of the second generation were also injected from the ninth to the thirteenth day. Several experiments were made for the determination of tumor growth in each of these four kinds of mice receiving the treatment outlined. A series of mice served for each experiment (A1, A2, B1, B2, C1, C2, D) in each of the four kinds of animals. Each series was again subdivided into four classes according to the weight of the tumors on the ninth day, and each class again divided into several groups. Each column was again divided into two subcolumns; in subcolumn B is given the percentage growth of the various classes of tumors in each series. The definitions of the various classes according to the weight limits of the various tumors are given in the preceding paper.[6] Such a subdivision is necessary because the percentage growth of tumors within a certain period of time depends upon the size of the tumors at the beginning of that period. At the same time we have to consider that this statement holds good only if we calculate the average percentage growth of a number of tumors. There are quite a number of variations of unknown origin if we consider

[6] Fleisher and Loeb, *Jour. Exper. Med., loc. cit.*

the individual tumors. In subcolumn R is given the percentage growth of the various series of mice. This figure can be computed in various ways. We can compare the weight of all the tumors of a series on the ninth day and on the thirteenth day and determine the percentage increase in the sum of the weights (α). Variations in the number of tumors of various sizes on the ninth day will cause variations in the figures thus obtained, so that the results in different series are not quite comparable. Or we can multiply the average of the percentage increase in weight in each class with the number of tumors in each class, add the figures thus obtained, and divide the sum by the total number of mice used in that series (β). Inasmuch as the number of mice in each class, as far as the valuation of the result of the experimental interference is concerned, is of no importance, it might be of advantage to add the percentage increase in the various classes of each series and divide by the number of classes. This is the best method, if there are enough tumors in each class and if all the classes are represented in each series. This is, however, not usually the case. It will therefore be necessary to interpolate the figures for missing classes by determining the average corresponding to the character of the other classes in that series. If the other classes have a high figure we fill out the vacancy by a correspondingly high figure, determined on the basis of the other experiments, and correspondingly in the case of a low average in the other classes. Thus the error is smaller than if we should altogether omit the figure for one or several classes. In this manner the third figure, γ, was obtained. In the end we took the arithmetical mean of these three figures in each series. This is the fourth figure, δ.

There is a further source of individual deviations from the expected figures in the various series, in each class the percentage increase depending to some extent upon the average class weight on the ninth day, whether it was relatively high or low. If it was relatively high the percentage increase is diminished in conformity with the rule that the larger a tumor is on the ninth day, the less is the percentage increase during the next four days. In table II we give, therefore, the average weight of each class on the ninth day. Each class we divide into two subclasses, one (1) with a relatively

TABLE II.

Class a^7 = 350 mg. and more.
Class c = 225–345 mg.
Class d = 125–225 mg.
Class f = 25–125 mg.

Control (uninjected) mice.		Mice injected with colloidal copper 9th–13th dy.	Mice injected with colloidal copper 2d–5th and 9th–13th dys.		Mice inoculated with tumor from mice previously injected with colloidal copper. Injected 9th–13th dy.	
A		A1 c 286 d 176 f 53			c 247 d 173 f 51	
D a 367 c 240 d 158 f 92	c + 144% d1 + 219% d2 + 208% f1 + 160% f2 + 121%	A2 c 283 d 198 f 68	A2 c 270 d 162 f 87	1 f1 0 5 b2 175% 3 d1 66% 4 d2 136% 4 c1 85% 1 c2 40%		
B1 a 350 c 271 d 180 f 91	1 f1 133% 5 f2 398% 3 d1 175% 3 d2 210% 5 c1 115% 1 c2 138%	B1 a 462 c 281 d 177 f 106	B1 a 466 c 291 d 186 f 92	1 f1 210% 7 f2 165% 5 d1 220% 9 d2 172% 19 c1 197% 6 c2 106%		
B2 a 525 c 291 d 137 f 72	13 f1 460% 5 f2 207% 5 d1 183% d2 4 c1 147% 2 c2 169%	B2 a 446 c 297 d 156 f 92			B2 a 421 c 280 d 160 f 60	9 f1 281% 4 f2 183% 12 d1 261% 6 d2 161% 18 c1 110% 4 c2 86%
C1 a 532 c 276 d 190	1 d1 212% 2 d2 172% 8 c1 141% 4 c2 137%	C1 a 439 c 293 d 162 f 87	C1 a 447 c 291 d 180 f 84	5 f1 345 11 f2 244 8 d1 291 11 d2 244 10 c1 170 6 c2 182		
C2 d 143 1 68	21 f1 321% 14 f2 263% 14 d1 167% 1 d2 113%	C2 a 476 c 284 d 156 f 73			C2 a 447 c 291 d 180 f 84	4 f1 206% 14 f2 310% 18 d1 314% 4 d2 148% 12 c1 151% 11 c2 170%

low and the other (2) with a relatively high class weight. We see that, wherever the number of tumors in both subclasses is suffi-

[7] Each class is divided into two subclasses, the dividing line between these subclasses being in each case the mean between the highest and lowest weight of that class.

ciently high, the lighter tumors have usually a greater percentage growth than the heavier tumors of the same class. Wherever the average weight of a certain class is therefore abnormally low we may expect that the percentage increase is relatively too great as compared with the increase of corresponding classes in other series. We made, therefore, a correction in the figure for class f of the first series in column 4 of table I. Here the average weight of the class is only 51. In this case we deducted about 50 per cent. from the actual percentage increase in weight. The average weight of the tumors on the ninth day in the various series of column 4 is relatively low. It was not essential to make any further corrections. In column 4 of table I we give under γ this corrected figure; in determining δ in this series we also made use of the corrected figure. There exists another factor which is responsible for certain variations in the individual experiments; namely, spontaneous retrogressions which may take place in class f. The number of spontaneous retrogressions in class f may vary; and this accounts for the relatively low average for class f in experiment D. In this experiment twenty-four tumors grew distinctly and twelve grew either very much below the average, or did not grow at all. This is the only experiment where this factor is of any significance.

On the whole, the figures designated γ in table I seem to be best for comparison of the percentage increases in weight in the different series of experiments.

A study of the averages of the various series as given in the summary in table I shows: (1) That the average percentage increases determined according to the different methods used by us give similar results; the greatest variation is found in column 4. (2) We find that four injections of colloidal copper retard the percentage growth of the tumors noticeably. If we adopt for comparison the figures γ and δ, uninjected control tumors grow 174 per cent. (180 per cent.), while tumors of mice injected with copper grow 110 per cent. (107 per cent.). (3) However, if we immunize mice by giving them four preliminary injections of colloidal copper from the second to the sixth day the inhibiting effect of the colloidal copper disappears, perhaps not altogether, but it certainly becomes insignificant; the respective figures for the immunized mice are now

167 per cent. (165 per cent.), which are only slightly below those of the control mice. (4) The fourth column shows a still more decided immunity of tumors of the second generation which had been subjected to injections of colloidal copper in the former generation of mice. Here the corresponding figures are 189 and 206 per cent. respectively. These figures are even slightly higher than those of uninjected control mice. Whether this increase is due to the variable factors mentioned previously or to a slight stimulating effect of injections of colloidal copper in the second generation, we are not ready to decide, although we are inclined to accept, at least at present, the first interpretation. (5) If we compare in the summary the figures for the percentage increase of the individual classes, we find that copper is about equally active in all four classes and that the immunization also affects all four classes. (6) If we compare instead of the figures given in the summary the individual series included in the summary we see that the figures in the various series are consistent with those of the summary, although there exist individual variations, especially in series in which the number of mice used is relatively small. It is necessary in valuing the relative importance of the figures in the various series to consider the number of mice used in a series; the series with a larger number of mice show on the whole a much smaller deviation from the average than series with a smaller number of mice. It is furthermore advisable to compare series in the same experiment. The conditions in different experiments vary more than in the same experiment, the kind of mice used and the tumor used for transplantation being more similar in the same experiment than in other experiments.

Table III shows the results obtained by the first method, in which we estimated the growth of the tumors by palpation. Table I gives the results obtained by weighing in the same experiments. We see that on the whole the results obtained by both methods agree very well, although in a few classes the results do not agree completely. Table IV presents the results in different classes (a) of uninjected controls, (b) of animals injected with copper without and (c) with previous immunization. The results were obtained with the aid of the first method. We see again that all classes are about equally

TABLE III.

Normal (uninjected) controls.	Mice injected with colloidal copper from 9th–13th dy.	Mice injected with colloidal copper from 2d–5th and 9th–13th dys.	Mice inoculated with tumor from mice previously injected with colloidal copper. Injected from 9th–13th dy.
H a 2 +[8] c 9 + 1 − d 16 + 2 − f 22 + 1 −	A1 c 2 + 3 − d 7 + 2 − f 3 + 7 −		A1 c 15 + 3 − d 12 + f 19 + 2 −
D a 4 + c 8 + d 31 + 3 ? 1 − f 24 + 7 + 5 −	A2 c 3 + 3 − d 2 + 2 − f 9 + 14 −	A2 c 5 + d 6 + 1 − f 5 + 1 −	
B1 a 1 + c 6 + d 5 + 1 − f 5 + 1 −	B1 a 8 + 11 − c 11 + 19 − d 4 + 4 − f 3 + 1 −	B1 a 11 + 1 − c 18 + 3 − 5 d 11 + 2 − f 7 + 1 −	
B2 a 10 + c 6 + d 4 + 1 − f 15 + 3 −	B2 a 4 + 6 − c 7 + 7 − d 5 + 7 − f 7 + 6 −		B2 a 7 + 1 − c 17 + 5 − d 17 + 1 − f 10 + 3 −
C1 a 3 + c 11 + 1 − d 3 +	C1 a 7 + 7 − c 7 + 8 − d 7 + 7 − f 8 + 7 −	C1 a 7 + c 16 + d 18 + 1 − f 12 + 4 −	
C2 d 11 + 4 − f 29 + 6 −	C2 a 4 + 4 − c 11 + 9 − d 7 + 8 − f 11 + 12 −		C2 a 6 + c 18 + 4 − d 20 + 2 − f 17 + 1 −

affected as far as the retardation in growth through colloidal copper as well as the processes of immunization are concerned. We carried out one similar experiment with hirudin. The various samples of hirudin vary somewhat in their efficacy and the one at our disposal at that time was rather weak. We have further to consider that in the case of the hirudin the results are complicated by hemorrhages into the tumor.

[8] In tables III and IV, + = grew; − = did not grow; ? = questionable.

TABLE IV.

Growth and Non-Growth as Shown by Palpation.

Normal control.	Immunized.	Colloidal copper controls.
a 14 +	a 32 +	a 23 +
	2 —	29 —
c 25 +	c 90 +	c 36 +
1 —	15 —	39 —
	5 ?	
d 24 +	d 84 +	d 34 +
8 —	8 —	29 —
f 48 +	f 70 +	f 41 +
11 —	12 —	47 —

In this experiment fifty-three mice were kept as controls, without injections; sixty-eight mice received injections from the ninth to the thirteenth day, gradually increasing doses of hirudin being given, and sixty-nine mice received four injections from the second to the sixth day and then again from the ninth to the thirteenth day. On examining the mice on the thirteenth day we found many hemorrhagic tumors in the second lot injected only from the ninth to the thirteenth day. The hemorrhages were very much less in the third lot which had received preliminary injections from the second to the sixth day. The increase in weight was much more considerable

TABLE V.

Normal control mice.		Hirudin injected 9th–13th dy.		Hirudin injected 2d–6th and 9th–13th dys.	
53 mice.		68 mice.		69 mice.	
H 174%	a + 113%	139%	c + 126%	220%	c + 208%
194%	c + 143%	150%	d + 150%	203%	d + 237%
166%	d + 147%	147%	f + 166%	207%	f + 177%
178%	f + 259%	145%		211%	

in the third than in the second lot. The preliminary injections seem, therefore, to have exerted an immunizing effect. We are here confronted with the fact that while the tumors of mice receiving hirudin injections from the ninth to the thirteenth day grew less than the uninjected control mice, the mice of the third lot grew

TABLE VI.

	Injected 2d–6th dy. with:	Injected 9th–13th dy. with:		Controls receiving injections of colloidal copper or hirudin 9th–13th dy. only.	
A Injected 2d–6th and 9th–13th dys.					
	Copper	Copper 36 R	244 G[9] (87%)	131 R[9]	120 G (47%)
	Copper	Hirudin 28 R	9 G (24%)	16 R	3 G (16%)
	Hirudin	Hirudin 38 R	183 G (83%)	94 R	63 G (40%)
	Hirudin	Copper 28 R	30 G (52%)	23 R	27 G (54%)
B Injected before inoculation					
	Injected before inoculation with:	Injected 9th–13th dy. with:			
	Copper	Copper 3 R	53 G (95%)	33 R	41 G (56%)
	Hirudin	Hirudin 58 R	38 G (40%)	45 R	21 G (32%)
C Transplanted tumor injected after the preceding generation had been injected					
	Preceding generation injected with colloidal copper Copper series				
	Copper injected 9th–13th dy.	62 R	441 G (87%)	183 R	122 G (40%)
	Hirudin injected 9th–13th dy.	57 R	24 G (30%)	39 R	9 G (19%)
	Preceding generation injected with hirudin Hirudin series				
	Hirudin injected 9th–13th dy.	61 R	83 G (58%)	66 R	22 G (25%)
	Copper injected 9th–13th dy.	62 R	36 G (37%)	51 R	30 G (37%)
D Injected 9th–13th dy.					
	Copper	371 R	277 G (43%)		
	Hirudin	184 R	102 G (36%)		
	38% of these retrogressed Of these 38% about 80% resumed growth when injections were stopped after the 4th dy., and about 70% resumed growth if injections were continued for 6 or 7 dys.				

[9] G = tumors growing at the normal rate; R = tumors with retarded growth or no growth.

more than the control mice. This is probably due to the fact that the weight of the tumors increases after injections of hirudin on account of edema and hemorrhages into the tumor; the average of the percentage increase of the tumors in the hirudin series is therefore less than the figures indicate. We must, however, at the present time admit that possibly some accidental factors may in addition be of importance (table V).

We carried out further experiments in order to determine the specificity of the immunity conferred by a first set of injections. We wished to determine whether or not preliminary injections of hirudin weaken the action of later injections of colloidal copper, and *vice versa;* and also whether injections of hirudin or colloidal copper given before inoculation with the tumor have the same weakening action on the effect of later injections of hirudin or colloidal copper given nine to thirteen days after inoculation. If that should be the case the immunizing action would take place through some organ or tissue of the injected mouse, independently of the tumor cells. For these experiments which were made at an earlier stage of our work we used the first method of determining the growth exclusively. We added, however, wherever it was feasible the figures obtained in later experiments in which we used for other purposes both the first and second method combined. Table VI shows the results of these experiments. Part D shows that more than one half of the tumors injected with either hirudin or colloidal copper are more or less retarded in their growth as a result of the injections. There does not seem to be a marked difference between the action of colloidal copper and of hirudin. Part B shows that injections of colloidal copper given before inoculation of the tumor lower markedly the effect of subsequent injections of colloidal copper. In the case of hirudin this lowering effect was apparently present only to a very slight extent. Part A shows that injections of colloidal copper given from the second to the sixth day weaken the effect of injections given from the ninth to the thirteenth day and that the same holds good in the case of hirudin, but that a first set of injections of hirudin does not markedly influence a second set of injections of colloidal copper, and that a first set of injections of colloidal copper does not influence markedly a second

set of injections of hirudin. Part C shows that a first set of injections of colloidal copper or hirudin given in one generation of tumor mice from the second to the sixth and from the ninth to the thirteenth days weakens the effect of a later set of injections given in the following generation of tumor mice, and again that this effect is specific, colloidal copper immunizing the tumor cells only against colloidal copper, and hirudin only against hirudin. In the case of hirudin the immunizing effect is apparently not quite as marked as in the case of colloidal copper.

In appraising the value of the results obtained we must be aware that in the best case only the principal facts can be established with the aid of the first method. Slight quantitative differences have to be disregarded altogether. As far as experiments have been carried out by both methods the results agree. We may therefore conclude that experiments carried out so far indicate that the immunization produced through preliminary injections of hirudin or colloidal copper is a specific one, each protecting only against later injections with the same substance. And we may also conclude that injections of colloidal copper, and probably also hirudin, given before the inoculation with the tumor have also an immunizing effect against later injections. Immunity against the action of colloidal copper and hirudin depends therefore on an immunity acquired by the host organism as well as on an immunity acquired by the tumor cells themselves.

In the case of hirudin we have also other evidence that processes of immunization take place in the organism of the injected mice; five to six intravenous injections of hirudin given on consecutive days immunize the animals against the anticoagulating effect of hirudin.[10] This effect can be demonstrated *in vitro* as well as *in vivo*.

We have still to consider another possible explanation for the results which we obtained; namely, the possibility that a selection of tumors naturally immune takes place, rather than active immunization of the tumors. This interpretation cannot be accepted (1) because the tumors subjected to the influence of the substances and the control tumors took their origin from the same tumor a few

[10] Vera, M., and Loeb, L., *Jour. Biol. Chem.*, 1914, xvii, p. xxv.

generations back. It can further be excluded (2) because after injection with colloidal copper from the second to the sixth day the majority of tumors grew under the influence of injections of colloidal copper given from the ninth to the thirteenth day. An accidental selection can therefore not very well take place under these conditions, because the chance that naturally susceptible tumors are used for transplantation is as great as the selection of naturally immune tumors. (3) If we compare the influence of colloidal copper on tumors that developed after transplantation from tumors whose growth had been retarded after injections of colloidal copper, and on those that developed after transplantation of tumors whose growth had not been influenced by colloidal copper, we find that both series are equally immune against the action of colloidal copper.

Tumors that had been transplanted from tumors that had proven resistant to the influence of colloidal copper gave the following result: 31 grew normally and 3 were retarded in their growth under the influence of colloidal copper. Tumors that had been transplanted from tumors that had been retarded in their growth under the influence of colloidal copper gave this result: 38 grew normally and 3 were retarded in their growth. Tumors derived from tumors that had grown well under the influence of colloidal copper gave the following result after injection of hirudin: 10 were retarded and 4 grew well. Tumors derived from tumors that had been retarded in their growth under the influence of colloidal copper gave the following result: 10 were retarded and 8 grew well under the influence of hirudin. We may therefore conclude that the results we obtained are due to an immunization of the tumor cells and not to a selection of naturally resistant tumors.

Our results point to the conclusion that the acquired immunity is a specific one. If this is the case, we should be able to affect, through injection of hirudin, tumors that had been resistant to the action of colloidal copper. This we found to be the case. Tumors of mice that had been injected with colloidal copper from the ninth to the thirteenth day and had grown normally under the influence of this substance, were injected with hirudin from the thirteenth to the sixteenth day. Of 36 animals thus treated, the tumors

retrogressed in 6, were retarded in their growth in 19, and grew normally in 11 animals. Hirudin had therefore its usual effect on tumors after colloidal copper had previously failed to exert any influence on the same tumors.

We also had to consider the possibility that the immunization was due to the fact that after injections from the second to the sixth day after inoculation the general health of the tumor mice was less affected through subsequent injections from the ninth to the thirteenth day, and that in consequence of the better health of the mice the tumors grew better in animals having had the first set of injections. However, we found that the appearance of both sets of animals, those having received two sets of injections and those having received only one set, were equal. In a certain number of animals we determined the effect of the injections on the weight of the animals and obtained the following results:

After inoculation	Average weight of mice receiving colloidal copper 9th–13th dy.	Average weight of mice receiving colloidal copper 2d–6th and 9th–13th dys.
2 dys.	16.2 gm. (100 mice)	16.7 gm. (100 mice)
9 dys.	16.1 gm. (78 mice)	16.2 gm. (75 mice)
13 dys.	16.2 gm. (63 mice)	16.2 gm. (66 mice)

We see that there is very little difference in weight on the ninth and on the thirteenth day in the mice of the two series. During the first injections, from the second to the sixth day, the mice of the second series lost slightly in weight, 0.5 of a gram on the average. In this series the weight remained constant during the second set of injections, while it increased slightly (0.1 of a gram) in the series not receiving the first set of injections. We may therefore conclude that general conditions of health cannot account for the immunity that we noticed.

We saw that as a result of the presence of several variable factors the growth differs considerably in individual tumors. This necessitated the use of large numbers of tumors, in order to test the effect of various factors on tumor growth. We are conscious of the fact that results based on statistical methods do not possess the same degree of certainty as results of experiments in which individual variations do not occur. On the other hand, the number

of tumors observed by us is unusually large. We lay at the present time the main emphasis on the publication of the actual results obtained in this as well as in the preceding communication, without considering the conclusions as yet as more than probable. However, we hope that the opportunity will soon present itself to us to carry out another series of similar experiments, and we shall then state whether or not the further experiments confirmed our previous work. With this understanding, implying the restrictions mentioned, we may point out some of the further conclusions that can be drawn from our work.

1. Our work shows that through injections of solutions of colloidal metals changes take place in the body which tend to counteract the further efficacy of the colloidal metal. It is probable that as the result of these reactions the colloidal solution itself within the body is changed to such an extent that it is less efficacious.

2. If, as our experiments seem to indicate, the immunity is in part localized in the tumor cells and if this acquired immunity is transferred to successive cell generations, and especially if this immunity is a specific one, it is probable that the various substances that we use attack the tumor cells directly and act only indirectly through changes in the circulation or in the blood vessels of the tumor or of its environment. The latter action may, however, exist side by side with the direct one on the cells. In a former publication[11] we have already drawn attention to this possibility.

3. Such processes of immunity as those that we observed would be an important factor in the practical application of chemotherapy. They would tend gradually to diminish the efficacy of the substances we use, provided that we do not apply substances causing a rapid destruction of the tumor. On the other hand, the specificity in these processes of immunization would make it possible to counteract the injurious influence of immunization established against one substance, by using new substances after the usefulness of the first substance has been noticeably diminished.

[11] Loeb and Fleisher, *Jour. Am. Med. Assn., loc. cit.*

SUMMARY.

1. Through a set of preliminary injections of colloidal copper or hirudin, given from the second to the sixth day after transplantation, we can weaken the effect of injections of the same substances given from the ninth to the thirteenth day after transplantation.

2. Injections of colloidal copper given before the transplantation of the tumor lead to a weakening of the effect of injections given from the ninth to the thirteenth day after transplantation.

3. The same result can be obtained if we inject colloidal copper or hirudin into mice from the second to the sixth and from the ninth to the thirteenth day after transplantation, and use these tumors at the end of the series of injections for further transplantation into another set of mice; the developing tumors are more resistant to the action of colloidal copper or hirudin than new control tumors.

4. We may therefore conclude that the immunity has two sources: (a) it is based on changes taking place somewhere in the host organism; and (b) it is localized in the tumor cells themselves which transfer this immunity to the following generations of tumor cells.

5. The immunity acquired against colloidal copper does not protect noticeably against the action of hirudin, and *vice versa*. The immunity is therefore specific.

6. We discuss certain general conclusions which may be drawn from these experiments. We expect to test the validity of these results in further experiments.

DO SUBSTANCES INHIBITING TUMOR GROWTH EXERT A RETARDING INFLUENCE ON THE REGENERATION OF THE SKIN?*

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In a series of studies carried out in this laboratory[1] the effect of various substances on tumor growth has been determined. Several substances, especially colloidal copper, hirudin, nucleoprotein, and casein were found to be active.

In order to contribute to the understanding of the mechanism of these substances, it was considered of interest to study their influence on other processes besides tumor growth, and at Dr. Loeb's suggestion I studied the effect of these substances on wound healing in the mouse.

The problem that I undertook was to determine if possible whether any change, macroscopic or microscopic, could be produced through the intravenous injection of these substances in a wound in the process of healing.

The technique adopted consisted in excising in each experiment a small piece of skin from the back of each of eight mice, care being taken to make excisions of about the same size. At first the wounds were protected by a colloidin dressing, but later this was omitted as the wounds seemed to heal better without this covering; and when the mice were kept in separate boxes no noticeable infection followed and the wound healed normally.

On the third day after the wound was made, four of the mice were injected through the vein of the tail with the substance to be tested, and injections were repeated on four successive days. The mice were killed on the eighth day and the wound with the surrounding skin was excised for microscopic study.

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[1] Fleisher, M. S., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 503.

The four control mice were either not injected at all or were injected intravenously with normal salt solution or distilled water. They were also killed on the eighth day and the wound with its surrounding skin was excised for microscopic study.

When a mouse died or was killed by the injection at an earlier date, the wound was excised and at the same time a control animal was killed and its wound excised for study.

Altogether twelve experiments were carried out with the four substances active in the case of mouse tumors. The wounds which were excised on the eighth day showed no difference macroscopically. Microscopically it was found that the regeneration of the epithelium, as well as of the connective tissue and blood vessels, was approximately the same in control mice, and in the mice injected with the various substances. We may therefore conclude that none of the substances mentioned has a noticeable influence on wound healing.

CONCLUSIONS.

Repeated injections of colloidal copper, hirudin, nucleoprotein, and casein, which have a definite retarding influence on tumor growth, given to mice during the process of wound healing do not produce any noticeable influence on the course of regeneration.